Study of *Ascl1* function in the neurogenic lineage of the adult mouse hippocampus

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Declaration of authenticity

The work presented in this thesis has been completed in the laboratory of François Guillemot in the Division of Molecular Neurobiology at the National Institute for Medical Research, currently The Francis Crick Institute, Mill Hill Laboratory. I, Jimena Andersen, confirm that this work is my own. Where information has been derived from other sources, this has been indicated in the thesis.

Processing of tissues for immunohistochemical analysis (perfusion, vibratome sectioning and staining) was done in its majority by Angeliki Achimastou (former research assistant in the laboratory). Laser capture microdissection (LCM) analysis in the Results sections 3.3.2, 4.3.2 and 4.3.4 was performed by Ayako Ito (former post-doc in the laboratory). ChIP-seq analysis referred to in Chapter 6 and described in Appendix 1 was performed by Ben Martynoga (former post-doc in the laboratory). Finally, isolation of RGLs by FACS was done in collaboration with Noelia Urbán (current post-doc in the laboratory).

Abstract

The adult mammalian brain is a highly plastic structure capable of cellular and molecular remodelling in response to its interactions with the outside world. The addition of new neurons to the hippocampus throughout life is one of the most striking manifestations of this plasticity. New neurons here are generated from a population of stem cells that, although existing primarily in a dormant or quiescent state, they can become activated upon the reception of neurogenic signals. How stem cells integrate these signals from the environment to ultimately control neuronal production is currently under investigation.

During embryonic development, transcription factors of the basic helix-loop-helix family promote progenitor proliferation and differentiation to ensure the production of neurons in correct numbers and at the correct positions. We found Ascl1, a proneural factor in this family, to be expressed by stem cells of the adult hippocampus when in an active state. Here we used pharmacological and genetic approaches to show that Ascl1 expression is rapidly induced in response to neurogenic stimuli, and that deletion of this factor with a conditional inactivation approach results in an inability of stem cells to respond to signals and exit their quiescent state. Moreover, by examining the genes deregulated in *Ascl1*-deleted stem cells, we show that Ascl1 promotes the proliferation of hippocampal stem cells by directly regulating cell cycle regulatory genes, among which the cyclin D genes are of great importance.

The data presented here supports a model whereby Ascl1 acts as a central factor in adult hippocampal stem cells to integrate both stimulatory and inhibitory signals and translate them into a transcriptional programme that controls stem cell activity. With this work we also highlight that understanding how Ascl1 is regulated will contribute, in the future, to the development of stem cell therapies for the treatment of neurological disorders.

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Abbreviations

Aβ	β-amyloid
AD	Alzheimer's disease
AH-NSC	Adult hippocampus-derived NSC
aNSC	Activated NSC
Ascl1	Achaete-scute complex homolog-like 1
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix-loop-helix
BLBP	Brain lipid-binding protein
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
bp	Base pairs
BrdU	5-bromo-2'-deoxyuridine
CA	Cornu ammonis
CBP	CREB-binding protein
Ccn	Cyclin
CDK	Cyclin-dependent kinase
CH	Cortical hem
ChIP-seq	Chromatin immunoprecipitation sequencing
CKI	Cyclin-dependent kinase inhibitor
CNS	Central nervous system
cKO	Conditional knock-out
DAPI	6-diamidino-2-phenylindole
DAVID	Database for annotation, visualization, and integrated discovery
DCX	Doublecortin
DEPC	Diethylpyrocarbonate
DG	Dentate gyrus
Dkk1	Dickkopf-related protein 1
Dll-1	Delta-like 1
DNA	Deoxyribonucleic acid
DNE	Dentate neuroepithelium

dpi	Days post injection
E	Embryonic day
EC	Entorhinal cortex
ECS	Electroconvulsive seizure
EGFR	Epidermal growth factor receptor
FACS	Fluorescence-activated cell sorting
fl	Floxed
FLP	Flippase
FMRP	Fragile X mental retardation protein
FXR2	Fragile X relative protein 2
Fzd1	Frizzled 1
GABA	γ-aminobutyric acid
GBM CSC	Glioblastoma cancer stem cell
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GL	Granule layer
Glast	Glutamate aspartate transporter
GO	Gene ontology
GREAT	Genomic regions enrichment of annotations tool
GSK3β	Glycogen synthase kinase 3 β
HD	Huntington's disease
HDAC	Histone deacetylase
hESC	Human embryonic stem cell
HLH	Helix-loop-helix
HNE	Hippocampal neuroepithelium
HSC	Hematopoietic stem cell
Id	Inhibitor of differentiation
IGF	Insulin-like growth factor
i.p.	Intraperitoneal
IPC	Intermediate progenitor cell
iPSC	Induced pluripotent stem cell
ISH	*In situ* hybridisation
KA	Kainic acid

LCM	Laser capture microdissection
MCM2	Minichromosome maintenance complex component 2
MGE	Medial ganglionic eminence
ML	Molecular layer
miR	microRNA
mRNA	Messenger RNA
MTLE	Model of temporal lobe epilepsy
NB	Neuroblast
Neurog	Neurogenin
NFIX	Nuclear factor 1/X
NMDA	N-methyl-D-aspartate
NPC	Neural progenitor cell
NSC	Neural stem cell
OB	Olfactory bulb
Olig2	Oligodendrocyte lineage transcription factor 2
P	Postnatal day
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated protein
PD	Parkinson disease
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
Prox1	Prospero homeobox protein 1
PSA-NCAM	Polysialylated neural cell adhesion molecule
PTEN	Phosphate and tensin homolog
qNSC	Quiescent NSC
qPCR	Quantitative polymerase chain reaction
Rb	Retinoblastoma
RGL	Radial glia-like
RMS	Rostral migratory stream
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
Rrm2	Ribonucleotide reductase M2
RT-qPCR	Reverse transcription qPCR

SEM	Standard error of the mean
SEZ	Subependymal zone
sFRP3	Secreted frizzled-related protein 3
SGZ	Subgranular zone
Shh	Sonic hedgehog
shRNA	Short hairpin RNA
SVZ	Subventricular zone
TA	Transition astrocyte
TAM	Tamoxifen
Tbr2	T-box brain 2
TSS	Transcriptional start site
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT	*Wild type*
YFP	Yellow fluorescent protein

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And I find myself, on the last day before submission, staring at the screen, struggling to find the right words to thank everyone that has taken part in this process. It has been long. It has definitely been hard. But it has also been incredibly fun and rewarding, and it has only been possible because I had the right people by my side.

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"It was the happiest moment of my life, though I didn't know it. Had I known, had I cherished this gift, would everything have turned out differently?"

Orhan Pamuk, The Museum of Innocence

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A Carmen y Florencia por la ambición y dedicación

Chapter 1 Introduction

Adult mammalian brains were long believed to be post-mitotic structures with little or no regeneration ability. However, the identification of neural stem cells (NSCs) that are able to self-renew and differentiate to give rise to all major neural lineages has shown adult brains to be highly plastic systems (Temple, 2001). Two main germinal zones have been identified where new neurons are born throughout adulthood: the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the walls of the lateral ventricles (Zhao et al., 2008). The ability of NSCs in these regions to promote the generation of new neurons throughout life depends on a tight balance of stem cell maintenance and proliferation (Schwarz et al., 2012). The control of this balance is achieved through the combinatorial activity of complex intrinsic programmes that regulate stem cell activity, and of extrinsic neurogenic signals that arise in the specialized microenvironment these cells are embedded in and with which they form a functional unit (Suh et al., 2009, Doetsch, 2003).

In this chapter I will summarise the present knowledge in the field of adult neurogenesis. I do not intend to be exhaustive, but to present those ideas that will be relevant for the analysis and understanding of the original work that I will present in the following chapters. I will start this section by presenting a general introduction of the topic and how it fits within a social and scientific context. Next I will move on to describe, in more detail, the process of neurogenesis in the hippocampus and how it is regulated by the environment. This will be followed by a description of the mechanisms that control the neurogenic programme, what will be the main focus of the chapter. Finally, I will present a short synopsis of the proneural factor Ascl1, why we think it might be important in the process of neurogenesis and how it might fit into the picture. The main goal in this section is to show a picture of a process that is intricately complex and that is influenced by an innumerable number of factors. Moreover, I aim to show a picture where all these factors remarkably come together to perform precise functions, and where the failure to do so results in devastating neurological disorders.

1.1 An introduction to the field

1.1.1 Brief historical perspective

If asked to think of a mammalian tissue with regenerative capacity, the brain would certainly not be our first choice. Blood, skin or intestines would most likely pop into our heads instead (Poss, 2010). Why this is the case can probably be briefly summarised in this quote from the early nineties by Santiago Ramón y Cajal:

> "Una vez que el desarrollo ha concluido, las fuentes de la regeneración de los axones y dendritas se agotan irrevocablemente. Preciso es reconocer que, en los centros adultos, las vías nerviosas son algo fijo, acabado, inmutable. Todo puede morir, nada renacer." (*Once development has ended, the sources of growth and regeneration of the axons and dendrites dry up irrevocably. In the adult centres, the nerve paths are something fixed, finished, immutable: everything may die, nothing may be regenerated*.) (Ramón y Cajal, 1928)

The brain, as described in this quote from the father of modern neuroscience, was believed to be a stable and "immutable" structure until not long ago. Neurons, the main cellular constitution of the brain, are post-mitotic, and therefore unable to divide. This, added to the failure to recognise the existence of NSCs in adult brains, led to the inevitable conclusion that neurons are incapable of regenerating.

The first mention of neurogenesis in the rodent adult hippocampus came from Joseph Altman in 1963 (Altman, 1963), when he labelled cells in adult rats with a thymidine analogue to show that indeed there were regions of proliferative activity and therefore, the possibility of new neurons being formed in adult structures. Later studies by Michael Kaplan in the adult olfactory bulb (Kaplan and Hinds, 1977) and by Fernando Nottebohm in brains of songbirds (Paton and Nottebohm, 1984) supported this notion. These were, however, discoveries made ahead of their time. The scientific community was not ready to believe this to be a true phenomenon. It was only after the discovery of NSCs in the SVZ (Reynolds and Weiss, 1992), and in the hippocampus (Palmer et al., 1995), as well as after the demonstration of neurogenesis taking place in adult human brains (Eriksson et al., 1998), that the idea of "new neurons for old brains" (Kempermann, 2011a) was finally accepted.

Adult neurogenesis research gained momentum from this time, with publications concerning adult NSCs growing exponentially in the last 20 years. The increasing need of new brain cells for the treatment of numerous neurological disorders, together with the finding that NSCs, especially in the hippocampus, are regulated by physical and cognitive activities and that therefore have biological and functional relevance (see below for details), made this a very promising area of research. Understanding the cellular and molecular mechanisms that regulate endogenous neurogenesis opens the doors to the exciting possibility of using the brain's own reservoir of stem cells as a potential therapeutic strategy (Taylor et al., 2013, Einstein and Ben-Hur, 2008).

1.1.2 Definitions: What is adult neurogenesis?

Adult neurogenesis, in its most general definition, is the production of new functional neurons in the adult brain (Kempermann, 2011a). A neurogenic region is, therefore, an area in the brain where new neurons are generated; and it normally implies 1) that precursor cells are present, and 2) that these precursors are surrounded by a special microenvironment or neurogenic niche that is permissive, i.e. that allows neurogenesis to take place. In the adult brain two main neurogenic regions exist: the hippocampus and the olfactory system. These are populated by a subset of undifferentiated precursors, the NSCs, that retain the ability to proliferate and self-renew, and are capable of giving rise to both neuronal and glial lineages (Shi et al., 2008)

While the precursors contributing new neurons to the olfactory system reside in the subventricular or subependymal zone (SVZ or SEZ) in the walls of the lateral ventricles, precursors generating the neurons that will contribute to the hippocampal formation are located in the subgranular zone (SGZ) of the dentate gyrus (DG, Figure 1-1). In both cases, a population of NSCs or radial glia-like cells (RGLs) will enter the cell cycle, from a relatively quiescent state, to proliferate and, if dividing asymmetrically, give rise to more committed progenitors that will rapidly divide to amplify the number of neurons produced (Zhao et al., 2008). Note that I will be using the term neural precursor when referring to all dividing cells in the lineage with some capacity for differentiation, including the primitive NSCs, while I will make use of

the term progenitors, instead, when only referring to the more committed population of cells that is derived from NSCs.

Despite sharing important features during the process of neurogenesis, a phenomenon that grants them the allocation into the same category, numerous differences between the SVZ and the SGZ exist. To begin with, at least in rodents, there is a dramatic difference in the number of neurons generated in each region, with the SVZ having a many-fold higher production compared to the DG (Kempermann, 2011a). Moreover, while precursors in the hippocampus generate excitatory granule cells that remain in the vicinity of their birthplace (Kempermann et al., 2004), those of the SVZ generate various types of interneurons that migrate over a long distance along the rostral migratory stream (RMS) to the olfactory bulb (Lledo et al., 2008). Consequently, the newly generated neurons in these regions play rather dissimilar roles, and respond differently to the various physiological demands of the organism and to threatening pathological insults.

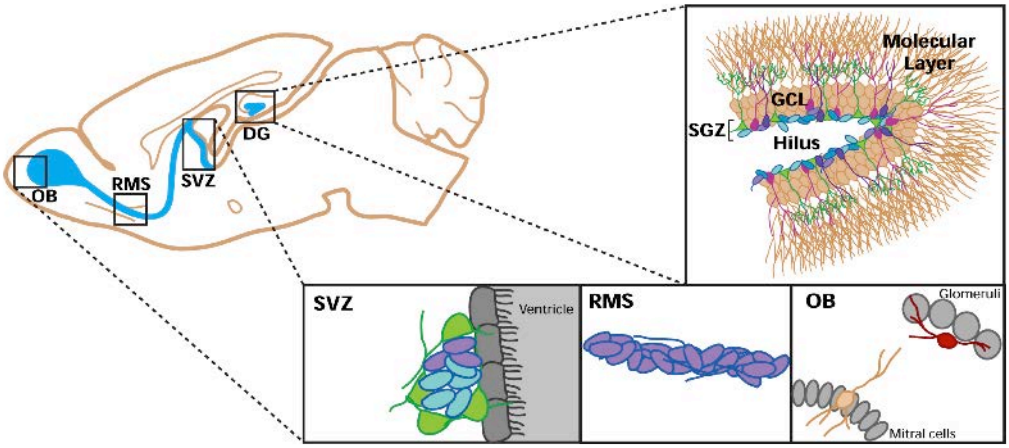

Figure 1-1 Neurogenic regions in the adult mouse brain

Sagittal view of the adult mouse brain showing, in blue, the two main regions where neurogenesis occurs throughout life: the dentate gyrus (DG) of the hippocampus, and the subventricular zone (SVZ). In the hippocampus, stem cells (green) reside in the subgranular zone (SGZ), at the base of the DG facing the hilus, and they extend radial processes along the granule layer or granule cell layer (GCL). Upon division, stem cells give rise to intermediate progenitors (light blue), which will in turn divide and generate first neuroblasts (blue) and finally immature (purple) and mature neurons (pink). Mature granule cell neurons extend their dendrites to the molecular layer to receive cortical input. In the SVZ, stem cells (green) reside in the wall of the lateral ventricles underneath the ependymal layer (grey). These also give rise to intermediate progenitors (light blue) and neuroblasts (purple). Neuroblasts migrate out of the SVZ, and they do so in chains that reach the olfactory bulb (OB) along the rostral migratory stream (RMS). In the OB, neuroblasts mature into functionally distinct neurons (red and peach). (Figure reproduced with permission from Johnson et al., 2009)

Excluding the hippocampus and the SVZ, the rest of the adult brain is considered non-neurogenic under physiological conditions, and this is because, even if dividing cells are found in other areas of the brain in large numbers, these do not give rise to functional neurons. Numerous reports, however, have claimed neurogenesis to be a more widespread phenomenon, and to take place also in other areas like the neocortex, the striatum and the hypothalamus (Dayer et al., 2005, Gould, 2007, Kokoeva et al., 2005). Furthermore, multiple studies have suggested that in response to pathological stimuli, otherwise "non-neurogenic" areas can become neurogenic (Magavi et al., 2000). Hypoxia and ischemia appear to be two insults that trigger this response (Arvidsson et al., 2002, Ohira et al., 2010).

1.1.3 Neurogenesis in the adult human brain

The discovery that new neurons are generated in the adult brains of humans by Peter Eriksson's analysis of tissue from deceased cancer patients in the late nineties (Eriksson et al., 1998), indeed helped overcome the scepticism with which adult neurogenesis was met and certainly facilitated the huge advancement in the field. The lack of techniques to study neurogenesis in the human brain, however, meant that the knowledge regarding the extent and the relevance of this process in humans was not extensive. In recent years, nonetheless, a few novel techniques have been introduced that have allowed for a better understanding of human neurogenesis in adult brains.

One of the approaches brought forward for measuring levels of neurogenesis in the human brain *in vivo* was the use of non-invasive imaging strategies, like that shown by Manganas and colleagues (2007), which used magnetic resonance spectroscopy to measure a biomarker associated with neurogenesis in the hippocampus. Another more recent and innovative approach used carbon dating to measure neurogenesis in humans relying on elevated levels of ^{14}C in genomic DNA following terrestrial nuclear bomb testing. With this technique, the group led by Jonas Frisén, was able to show that in adult humans there is a significant turnover of hippocampal neurons, with 700 of them being generated per day (Spalding et al., 2013).

Therefore, it is now clear that new neurons are born in the human hippocampus and that they may contribute to brain function. However, the evidence supporting neurogenesis in the adult human SVZ is less conclusive. Some groups report the presence of neurogenesis in this area in humans as well as the existence of a chain of migratory neuroblasts as seen in the rodent RMS (Curtis et al., 2007), while others find no detectable addition of neurons in the olfactory bulb and show that this is almost completely absent after childhood (Sanai et al., 2011). In view of this controversy, Frisén's group has used the carbon dating technique to investigate the presence of neuron-producing progenitors in the lateral ventricles of humans. What they found is that while there are neuronal precursors present in the SVZ of humans, neuroblasts generated here appear to migrate, in their majority, to the striatum. Moreover, they find that, very interestingly, these adult-generated striatal neurons are preferentially depleted in Huntington's disease patients (Ernst et al., 2014; see also section 1.3.6).

No doubt new *in vivo* approaches to study neurogenesis in adult human brains will clarify a lot of the questions that remain regarding the relevance of the newly born neurons. These, however, will likely be complemented with novel *in vitro* tools that facilitate the manipulation of neuronal progenitors. The use of human pluripotent embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), for example, have revolutionised our understanding of the mechanisms governing stem cell maintenance and subsequent differentiation (Jessberger and Gage, 2014, Yu et al., 2014, Yu et al., 2013). In the near future, the use of cerebral organoids derived from human cells will also allow for the study of integration and functional connectivity within live neuronal networks (Lancaster et al., 2013, Pasca et al., 2014).

1.1.4 An evolutionary view of adult neurogenesis

In order to understand adult neurogenesis in a general context, it is important to very briefly consider how this trait might have evolved. Two main ideas have been postulated to answer this question. The first, and possibly the most widespread of these is based on the idea that neurogenesis in adult organisms was gradually lost during the course of evolution (Kaslin et al., 2008, Lindsey and Tropepe, 2006).

While in mammals adult neurogenesis is restricted to two telencephalic active regions, in non-mammalian vertebrates (fish, amphibians, birds and reptiles) proliferation and neuron generation occur extensively (Barker et al., 2011). The neuroscientist Pasko Rakic (2004) has suggested that this decrease in the extent of adult neurogenesis in mammals, and in particular in humans, might be due to a resistance to incorporate new neurons into an already established and mature network, and that strong suppressors of neurogenesis have evolved to restrain the potential of progenitors in the brain.

The alternative idea proposed by Gerd Kempermann (2012) is that neurogenesis in the adult DG might be instead a late-evolved trait, and that the plasticity conferred to the hippocampus by the addition of new neurons might have provided mammalian species with the ability to adapt to new and challenging environments. A number of arguments support this idea. First, even though the hippocampus is an ancient part of the brain, lower vertebrates do not have a DG and therefore lack a comparable functional structure (Treves et al., 2008). Moreover, when considering the proposed function of adult neurogenesis in rodents (see below), it becomes apparent that the flexibility to contextualise important pieces of information can provide a beneficial advantage to conquer new ecological niches (Glasper et al., 2012, Kempermann, 2008). Quantitative analysis of adult neurogenesis in more species, together with computational models that factor adaptation in will provide further evidence to prove or disprove these theories.

1.2 Neurogenic niches in the adult brain

Increasing understanding of the mechanisms controlling self-renewal and differentiation have made it apparent that stem cell function, and particularly cell fate decisions are under the influence of the microenvironmental niche the stem cells reside in. This role is highlighted by transplantation experiments where, for example, both DG-derived progenitors and neonatal cerebellar astrocytes are able to generate olfactory interneurons when grafted into the SVZ, while progenitors derived from the SVZ differentiate into glial cells if transplanted into non-neurogenic regions of the brain (Seidenfaden et al., 2006, Suhonen et al., 1996, Zheng et al., 2006). In the adult brain, some common components of the neurogenic niches that are known to

regulate stem cell function are blood vessels, mature residing astrocytes, the extracellular matrix, and several innervating neurotransmitter systems, among others (Doetsch, 2003). Understanding how each of these components regulates adult stem cells will be critical for better comprehending the mechanisms by which these cells are kept in a tight balance between self-renewal and differentiation and, ultimately, for the exploration of therapeutic applications.

1.2.1 The SVZ neurogenic niche

The neurogenic niche lining the lateral ventricles, the SVZ, has been extensively studied in past years and some defining features have started to emerge (Figure 1-2; Ihrie and Alvarez-Buylla, 2011). The SVZ, as the DG of the hippocampus, consists of three populations of lineage-related progenitors. The slowly dividing population of NSCs (B cells) lines the ventricle and gives rise to C cells, which correspond to transit amplifying progenitors that in turn divide to generate neuroblasts (A cells). These will migrate along the rostral migratory stream (RMS) to reach the olfactory bulb and integrate into the circuitry (Zhao et al., 2008).

B cells in the SVZ contact the ventricles through specialized apical processes that contain a primary cilium, while at the same time contacting blood vessels through long basal processes. In this way, B cells are exposed to both cerebrospinal fluid and blood-borne factors (Mirzadeh et al., 2008, Shen et al., 2008, Tavazoie et al., 2008). The influence of blood vessels on B cells is not restricted to vasculature-secreted soluble factors, though. Direct cell-cell contact between blood vessels and SVZ NSCs has been shown to maintain these in a quiescent state. Endothelial receptors ephrinB2 and Jagged1 work together to regulate stem cell behaviour by suppressing cell cycle entry and inhibiting differentiation, respectively (Ottone et al., 2014). This data suggests a model where contact with the vasculature keeps stem cells quiescent, while loss of this anchorage makes NSCs prone to differentiation in response to soluble factors released in the niche, and highlights a possible mechanism by which the neurogenic niche is able to promote opposite biological outcomes.

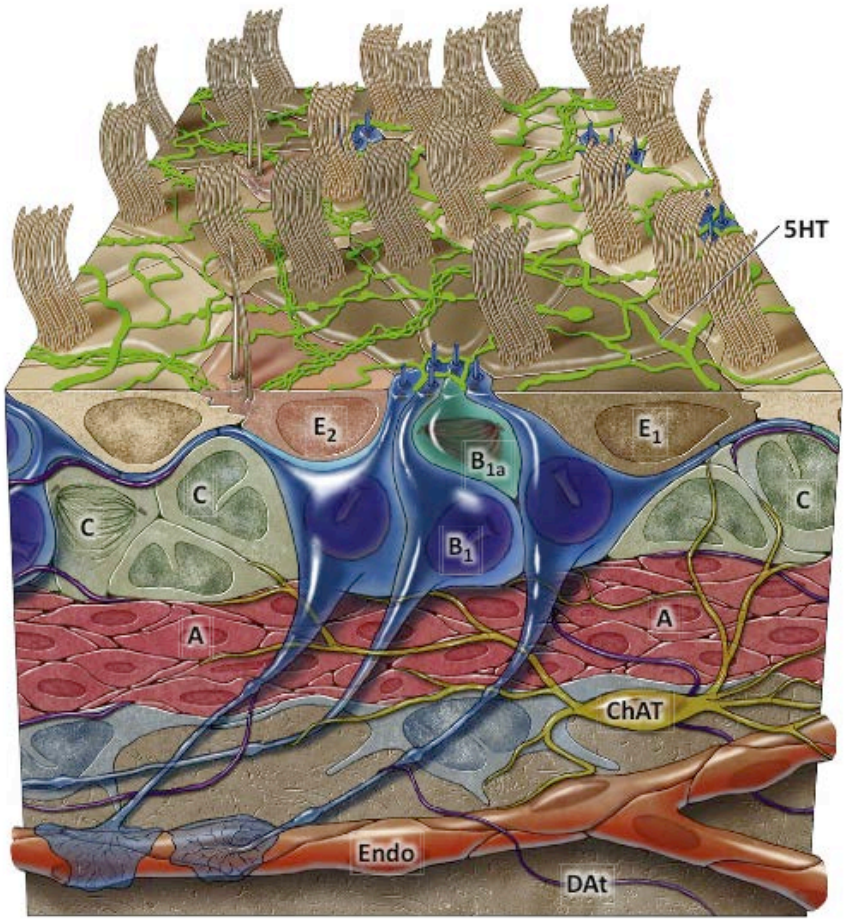

Figure 1-2 Schematic of the SVZ neurogenic niche

Stem cells in the SVZ (B_1, dark blue) reside just beneath the ependymal layer that lines the lateral ventricles. Ependymal cells (E_1 and E_2, brown) are multicilliated cells that form pinwheel structures on the ventricle surface. B cells contact the ventricle with an apical process that extends through the ependymal pinwheel structure. B cells also contact blood vessels (Endo, red) through basal processes. Once activated, B_1 cells (B_{1a}, light blue) give rise to transit-amplifying C cells (green), which, in turn, generate A cells or neuroblasts (red, shown here as a migrating chain). The SVZ receives innervation from a number of different neurotransmitter systems (e.g. serotonin, 5HT; dopamine, DAt; acetylcholine, ChAT). (Figure reproduced with permission from Lim and Alvarez-Buylla, 2014)

Another component that, also in relation with the blood vessels, has been shown to be important in maintaining stem cell function in the SVZ, is the astrocytes. Astrocytic processes contact all cell types in the SVZ niche. End-feet within these processes are closely associated to the walls of blood vessels, and regulate, like this, the induction and maintenance of the blood-brain barrier (BBB, Abbott et al., 2006). At sites where NSCs' basal processes contact the vessels, however, astrocytic end-feet are absent, thus modifying the BBB and exposing NSCs to blood-derived factors (Tavazoie et al., 2008). Moreover, clusters of proliferating B and C cells appear to be

associated with blood vessels at these sites where the BBB is leaky (Tavazoie et al., 2008), pointing at the regulation of progenitor cells of the SVZ by molecules in the circulation.

And finally, NSCs of the SVZ are found intercalated within ependymal cells, a single layer of multi-ciliated cells adjacent to the ventricle that form pinwheel-like structures around the apical processes of B cells (Mirzadeh et al., 2008). Ependymal cells synthesize molecules such as noggin, a bone morphogenetic protein (BMP) inhibitor, and platelet-derived growth factor (PDGF) that maintain the mitogenic character of stem cells (Jackson et al., 2006, Lim et al., 2000). All of these examples show the close connection that exists between stem cells and their niches, and how these associations are not merely anatomical, but that they also create functional links.

1.2.2 The DG neurogenic niche

In the case of the DG, the nature of the stem cell-niche interactions is less well understood, but some examples of their associations are increasingly coming to light. Progenitors in the SGZ have also been shown to bare close associations with blood vessels, the extracellular matrix and post-mitotic cells like neurons, astrocytes and microglia (Figure 1-3).

Astrocytes in the hippocampus represent one of the major contributors to the niche, as they appear to play a number of important and apparently opposite roles. For example, they have been shown to promote both progenitor proliferation and neuronal fate commitment by, in part, the secretion of Wnt molecules (Song et al., 2002, Lie et al., 2005; see also section 1.4.2.1), as well as the expression of ephrinB2 on their surface (Ashton et al., 2012). At the same time, astrocytes have been involved in the negative regulation of neurogenesis *in vitro* via a mechanism involving Jagged1 and Notch signalling (Wilhelmsson et al., 2012).

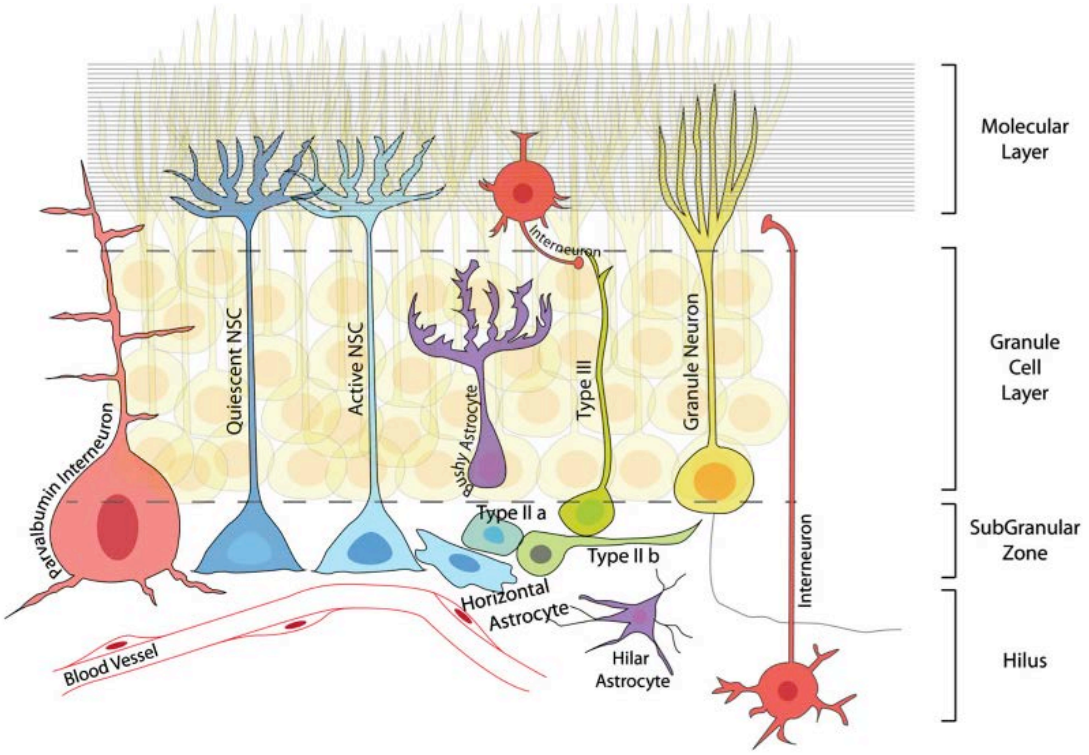

Figure 1-3 Schematic of the neurogenic niche in the DG

Stem cells in the DG (NSCs, dark and light blue), which can be in an active or quiescent state, reside in the subgranular zone. Horizontal astrocytes and intermediate progenitors (type II a and type II b, dark green) also reside here, while neuroblasts (type III, light green) and granule neurons (yellow) reside in the granule cell layer. Intermediate progenitors and NSCs are in physical contact with blood vessels in the DG. Astrocytes (purple) and interneurons (red) are located either in the hilus or granule cell layer and they are important constituents of the niche. (Figure reproduced with permission from Urbán and Guillemot, 2014)

Microglial cells are another cell type present in the niche that is important in DG neurogenesis. Microglia are the primary immune cells of the brain, and with their dynamic processes they interact with neurons, astrocytes and endothelial cells, among others, to constantly survey the local environment (Nimmerjahn et al., 2005). Phagocytosis of new-born DG cells by microglia provides a mechanism for maintaining a basal level of neurogenic output, and therefore maintaining hippocampal homeostasis (Sierra et al., 2010). Not only this, but microglial function in the niche also depends on the secretion of cytokines and chemokines that contributes to the balance of pro- and anti-inflammatory signalling that will either positively or negatively regulate neurogenesis (Carpentier and Palmer, 2009, Battista et al., 2006, Monje et al., 2003).

A few important examples also exist when considering physical contacts between neuronal precursors in the SGZ and components of the niche. The most significant of these is the intimate association of neuronal precursors and blood vessel endothelial cells. In a study carried out by Palmer and colleagues (2000), progenitors in the DG were found to be, as those in the SVZ niche, forming dense clusters adjacent to or surrounding small capillaries. Moreover, when studying the phenotype of cells incorporating 5-bromo-2'deoxyuridine (BrdU) after 2 hours of injection, they found 37% of BrdU immunoreactive cells to be of endothelial lineage, a discovery that led to the suggestion that neurogenesis is uniquely associated with the process of angiogenesis in the DG. The intriguing possibility of an intricate functional cross-talk between components of the nervous and vascular systems in the adult DG makes for an appealing hypothesis but has yet to be confirmed, as the finding of angiogenesis in this region of the adult brain has not yet been replicated (see Appendix 2 for a brief characterisation of angiogenesis in the DG, also performed as part of my doctoral work).

1.3 The adult mouse hippocampus

The hippocampus, or hippocampal formation, is a bilateral structure situated within the temporal lobes of the brain. It consists of four parts: the dentate gyrus, the cornu ammonis (or CA), the parasubiculum and the subiculum. The simple laminar pattern into which it is organised has made the hippocampus a central model system to record synaptic events, and as that, one of the most studied areas of the mammalian central nervous system (CNS; Li et al., 2009b, Neves et al., 2008). It is generally accepted that the hippocampus serves a crucial role in some forms of learning and memory. Lesions of the hippocampus in humans results in an inability to form new episodic memories, as was the case for the famous patient H.M., who underwent surgery to remove the medial temporal lobes as a way to treat intractable epilepsy (Scoville and Milner, 1957). Further studies using animal models have strengthen this understanding. Loss of hippocampal function both by controlled lesions and pharmacological or genetic inactivation of specific neuronal circuits have led to results involving a failure to learn or a loss of spatial or temporal memory, and have suggested to fit a model where the role of the hippocampus is to establish a spatio-temporal organization of memories (Eichenbaum, 2013).

1.3.1 Neuronal circuits in the adult hippocampus

The mammalian hippocampus consists of a trisynaptic core circuit that is organised in a unidirectional progression of excitatory pathways that links the DG, the CA3 and the CA1 (Figure 1-4; Li et al., 2009b, Kempermann, 2011a). The starting point of the trisynaptic hippocampal circuit is the entorhinal cortex (EC), from where, via the perforant path, most of the sensory information reaches the hippocampus. Afferents from layer II of the EC project through the subiculum to the dendrites of the granule cells in the outer molecular layer of the DG. Here, the axons of the granule cells form the mossy fibre tracts that will terminate on the dendrites of CA3 pyramidal cells. The last synapse in the circuit consists of a projection, termed Schaffer collateral projection, between the pyramidal neurons of CA3 to those in CA1. CA1 neurons finally return, through the subiculum, to convey sensory information to the EC, the cortical region of origin. Other pathways exist that add complexity to the concept of the trisynaptic circuit. The EC, for example, can bypass the DG and directly project to CA3 and CA1. Moreover, numerous types of local inhibitory interneurons modulate the neuronal activity of the DG mossy fibres; while a number of neurotransmitter systems project to the hippocampus from the rest of the brain. The hippocampus is, therefore, under the control of an extensive input system, which will undoubtedly have a profound influence on the regulation of neurogenesis in this region of the adult brain.

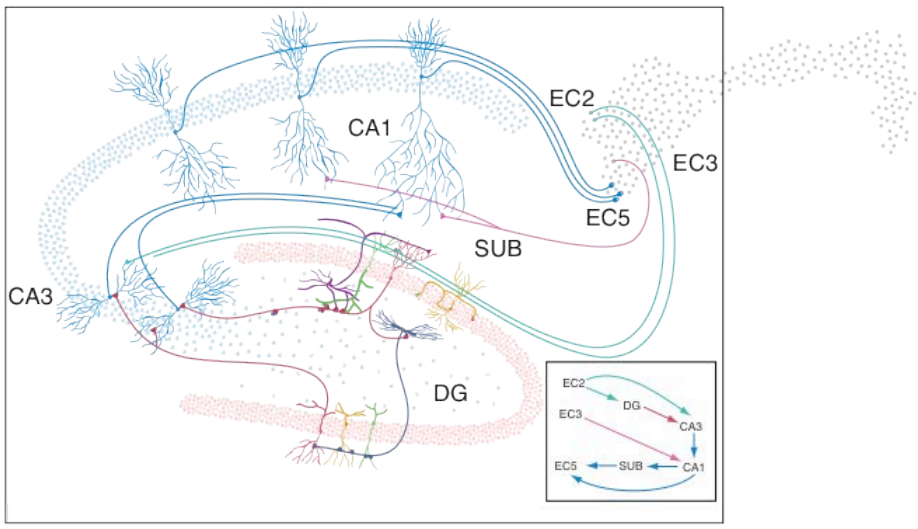

Figure 1-4 Schematic of the hippocampal network (legend next page)

Figure 1-4 Schematic of the hippocampal network

Sensory information reaches the hippocampus through the entorhinal cortex (EC). The EC axons then project through the subiculum (SUB) to the DG, and from here to the CA3. Finally, CA3 neurons project to CA1, from where sensory information returns to the EC (see text for more details). (Figure reproduced with permission from Li et al., 2009b)

1.3.2 Developmental origin of the hippocampal DG

Within the hippocampal formation, it is only in the DG that neural progenitors are found and new neurons are generated throughout life. A developmental point of view might give us a clue as to why the DG of the hippocampus is different from other regions of the adult brain. For this reason, here I would like to very briefly address how is the DG generated and where do stem cells in the adult originate from.

The neuroepithelium that gives rise to the hippocampal formation is a part of the dorso-medial region of the telencephalon and it consists of three different components, each unique in terms of morphology and molecular markers (Sugiyama et al., 2013). These are the hippocampal neuroepithelium (HNE), the dentate neuroepithelium (DNE) and the cortical hem (CH). The DNE, also called primary matrix, represents the first proliferative zone from where the first granule cells are generated at embryonic day 14.5 (E14.5). At late gestational stages (E17.5) progenitors migrate out of the DNE into the nascent DG, and they do so along a primordial radial glial scaffold that extends from the CH to the pial surface. Around birth, the main proliferative zone switches to the dentate area, including the hilar region and the blades of the granule cell layer, which are starting to be formed (Li et al., 2009a). At this time the primordial radial glial scaffold disappears, with a secondary scaffold developing approximately a week after birth (Brunne et al., 2010). From the second postnatal week, proliferation becomes limited to the SGZ of the DG, where it continues into adult life (For further information on each of the developmental steps and genetic regulators see Li and Pleasure, 2005).

The origin of the NSCs that will inhabit the SGZ of the adult hippocampus is still under examination. The prevalent hypothesis is that RGLs originate from hilar progenitors in the tertiary matrix that is formed at peri-natal stages (Brunne et al., 2010, Namba et al., 2005, Seki et al., 2014). What the relationship between the

progenitors in the tertiary matrix and those in the primary and secondary ones is not completely understood, though. A recent study reported that the stem cells responsible for persistent neurogenesis in the adult DG are derived during late gestation from sonic hedgehog (Shh)-responsive cells from a restricted region in the ventral hippocampus (Li et al., 2013). With this data a number of questions arise. For example, is there more than one pool of progenitors that give rise to adult NSCs? And if so, do they respond differently to different signals in the environment? Answers to these questions will also have implications in the search for stem cell heterogeneity during adulthood, but further studies will be needed to answer them.

1.3.3 Neuronal development in the adult DG

The formation of new neurons in the SGZ of the adult hippocampus is characterised by the progression of a multi-potent, self-renewing population of stem cells to mature functional neurons through a number of different stages, where cells are identified by their proliferative ability, their morphology and position in the DG, and by the expression of specific markers (Figure 1-5; Kempermann et al., 2004, Ming and Song, 2011).

The stem cell of the adult DG (type 1 cell or RGL) is a radial-glia-like cell, with its soma positioned in the SGZ and a single apical process that extends through the granule cell layer (GL). They share several features with astrocytes, such as electrophysiological properties, vascular end-feet, and the expression of the markers glial fibrillary acidic protein (GFAP), brain lipid-binding protein (BLBP) and glutamate aspartate transporter (GLAST). They can, however, be distinguished from astrocytes by their expression of Nestin, and their lack of the mature astrocytic marker S100β (Seri et al., 2001, Filippov et al., 2003, Kempermann et al., 2004, Brunne et al., 2010). Recently, a second type of type 1 cells, with short horizontal processes and higher proliferative ability, has been described. The lineage relationship of these cells and radial type 1 cells remains to be determined (Lugert et al., 2010).

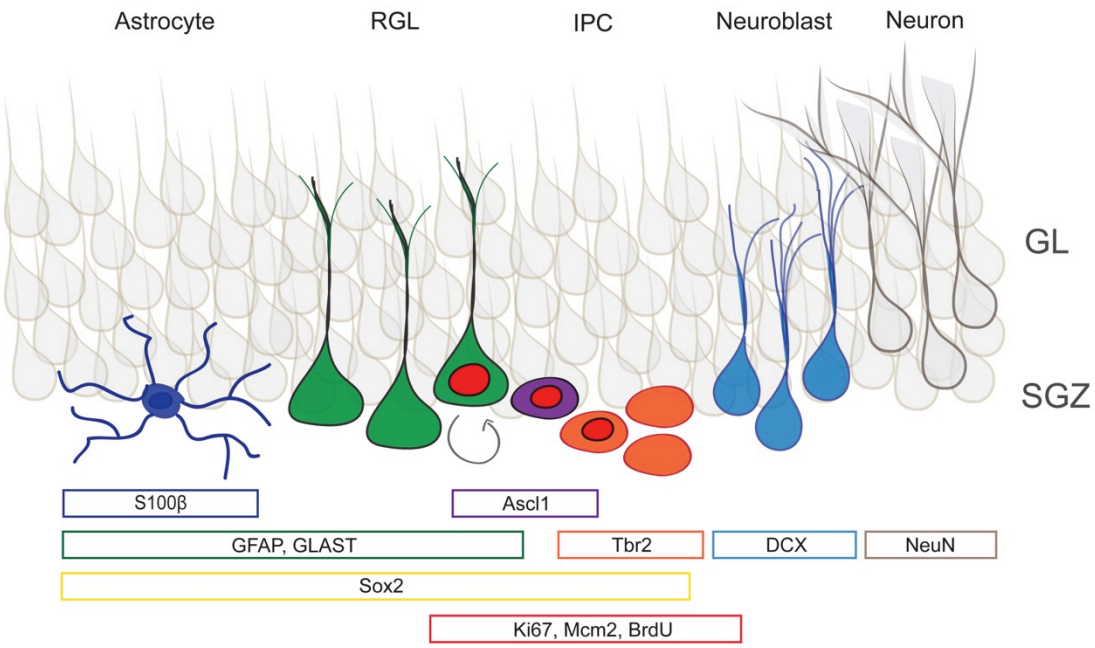

Figure 1-5 Neurogenic lineage and markers used to study neurogenesis in the adult DG

Stem cells or radial glia-like cells (RGLs, green) are positioned in the subgranular zone (SGZ) of the DG and have radial processes that extend along the granule layer (GL). They express the astrocytic markers GFAP and GLAST, but they do not express more mature astrocytic markers like S100β (mature astrocyte in dark blue). RGLs are found mainly in a quiescent state, but they will activate to give rise to the rest of the lineage. Intermediate progenitors (IPCs) are generated when RGLs divide asymmetrically. IPCs divide rapidly to amplify the lineage, and they are characterised by Ascl1 expression first (purple), and Tbr2 expression after (orange). Cell division can be detected using antibodies against Ki67 and MCM2, or by BrdU incorporation (red nuclei). All precursor cells express Sox2. IPCs generate neuroblasts (light blue), and finally mature neurons (light brown). These express the markers doublecortin (DCX) and NeuN, respectively, and can be found in the GL.

In a young adult murine brain, thousands of RGLs exist, but only a small proportion of them (1-5%) undergo cell division at one time, with the majority of them being in a relatively quiescent state (Kronenberg et al., 2003, Seri et al., 2001). At present, a number of models exist to explain the long-term potential and self-renewal capabilities of adult RGLs (Figure 1-6). Using population analysis, Encinas et al. (2011) came to the conclusion that RGLs undergo activation-dependent depletion throughout adulthood, and therefore do not possess long-term maintenance. According to this study, when RGLs exit quiescence and enter the cell cycle, they divide asymmetrically, three times on average, to generate new neurons before terminally differentiating into astrocytes. In conflict with this model, Bonaguidi et al. (2011) showed, by performing clonal analysis, that RGLs undergo several rounds of division to produce both neurons and astrocytes over a long period of time, and that

therefore RGLs are capable of self-renewal and multilineage differentiation. Yet another study by Dranovsky et al. (2011) has reported that RGLs in the adult hippocampus can accumulate, and that the lineage outcome is dictated by the animal's environment. In this model, an enriched environment can shift the RGL lineage toward a terminal neuronal population, while social isolation and irradiation result in an accumulating RGL population. The idea behind this form of neuronal plasticity is that accumulation of RGLs under deprived conditions would lead to increased neurogenesis in ameliorating conditions. The differences observed in the properties of RGLs in these studies have been attributed to differences in experimental designs, and particularly in the targeting and labelling of divergent RGL populations (Bonaguidi et al., 2012, Encinas and Sierra, 2012), pointing at a potential heterogeneity between apparently similar precursor populations.

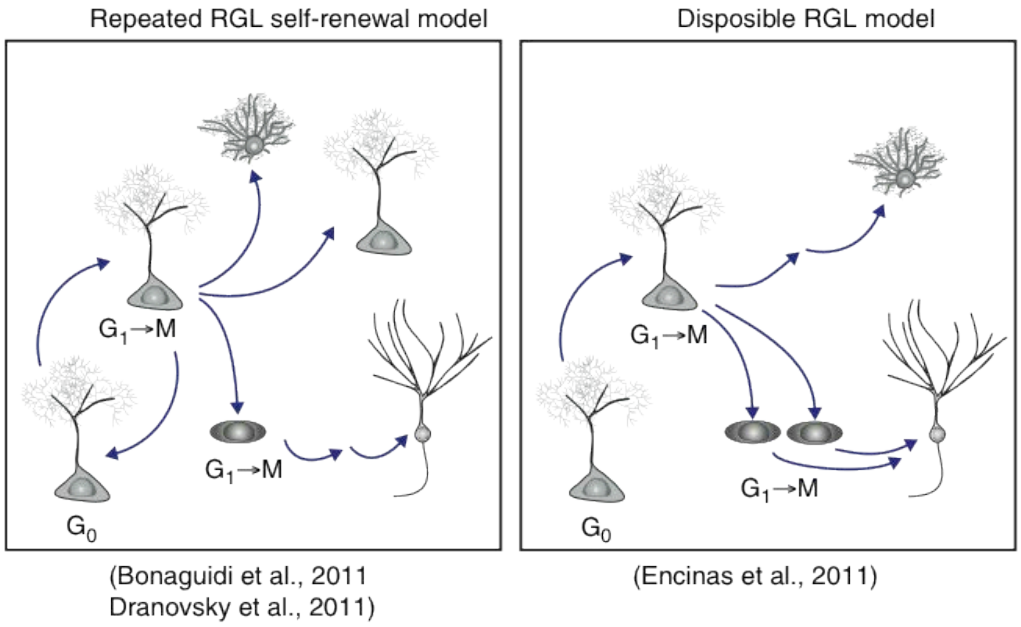

Figure 1-6 Models for stem cell behaviour in the adult hippocampus

Two main models have been proposed to explain RGL behaviour in the DG. Bonaguidi et al., 2011 propose a model where RGLs can divide symmetrically to generate additional RGLs, as well as asymmetrically to generate neuronal and astroglial lineages. Stem cells can, therefore, cycle between quiescent (G_0) and active states (G_1➔M). Similarly, Dranovsky et al., 2011 have proposed that RGLs are able to divide symmetrically and accumulate, but that the environment dictates this behaviour (opposed to a terminal neurogenic behaviour). In contrast, in the disposable RGL model proposed by Encinas et al., 2011, once activated, RGLs only divide a number of times before they terminally differentiate into astrocytes. In this model, therefore, activated RGLs cannot return to a quiescent state. (Figure adapted and reproduced with permission from Bonaguidi et al., 2012)

It is generally accepted, nevertheless, that once RGLs enter the cell cycle to undergo an asymmetric division, they give rise to transit-amplifying progenitor cells, type 2 cells or intermediate progenitors (IPCs). Morphologically, IPCs are small with short, tangentially-oriented processes, and they are normally located in clusters exclusively in the SGZ. One defining property of these cells is their highly proliferative ability, which accounts for the bulk of mitotic activity in the DG and for the large amplification observed in the lineage (Kronenberg et al., 2003). Two subgroups of type 2 cells can be distinguished: type 2a, or early IPCs, still express Nestin and also express the bHLH factor Ascl1; type 2b, or late IPCs, on the other hand, do not express Nestin and start expressing markers characteristic of neuronal commitment, namely, the T-box transcription factor Tbr2 and the bHLH transcription factor neurogenin 2 (Neurog2; Kempermann et al., 2004). Another characteristic marker expressed in all precursor cells, including RGLs, is Sox2, an HMG-containing transcription factor that is also broadly expressed by astrocytes throughout the nervous system (Suh et al., 2007).

Transition of IPCs into neuroblasts or type 3 cells involves great morphological changes and shows of functional differentiation. These cells are further advanced in the expression of neuronal features and express the immature neuronal markers prospero homeobox-1 (Prox1) and doublecortin (DCX) and the polysialylated neural cell adhesion molecule (PSA-NCAM, Lavado et al., 2010, Kempermann et al., 2004). At this stage, neuroblasts migrate to the GL and show a rounded nucleus with an apical dendrite, and whereas some of them retain the ability to proliferate, most of them become post-mitotic. Also, it is at this point when network connections are established and the selection for long-term survival occurs (Kempermann et al., 2004).

Finally, as neuroblasts migrate into the GL, they down-regulate DCX expression and up-regulate expression of more mature neuronal markers, such as NeuN and calretinin (Kempermann et al., 2004, Brandt et al., 2003). Features of mature neurons, like the expression of calbindin, are displayed at about 4 weeks of age, but new-born cells will continue to physiologically and morphologically change until up to 6-7 weeks of age, when they will become functionally indistinguishable from older granule cells (Jessberger and Kempermann, 2003, van Praag et al., 2002).

Retrovirus-mediated GFP-labelling of dividing cells has allowed for accurate morphological and electrophysiological analysis of adult-born neurons (Figure 1-7; van Praag et al., 2002, Zhao et al., 2006, Esposito et al., 2005). Such studies have shown that it is from an early post-mitotic stage, between 3 days post injection (dpi) and 14 dpi, during the phase of calretinin expression, that the largest part of the dendritic tree is built. During this time dendritic processes reach the outer edge of the molecular layer, while the axon fibres reach the CA3. Spine formation and growth starts taking place only once the first stage of dendritic and axonal growth is completed. It is also from this time that the major synaptic connections are generated (Zhao et al., 2006). In terms of functional maturation, it appears that neuronal differentiation in the adult hippocampus resembles that of its development. During the first week post-injection the cells are synaptically silent, and it is only after 2 weeks that the first γ–aminobutyric acid (GABA)-ergic synaptic inputs appear (Esposito et al., 2005). This first GABAergic input is excitatory and it drives further maturation and integration of the new-born neurons (Ge et al., 2006). By the fourth week glutamatergic input becomes detectable, coinciding with the display of mature characteristics like the presence of mature dendritic spines and the increased synaptic plasticity. Maturation is completed with the inhibitory response to GABA at approximately four weeks in neurons already possessing functional glutamatergic inputs (Esposito et al., 2005).

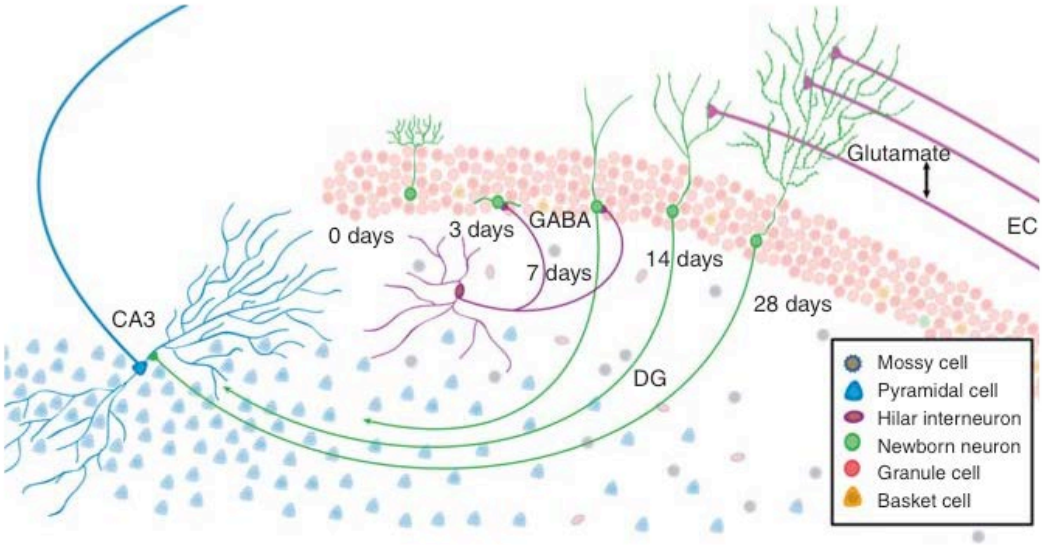

Figure 1-7 Time course of neuronal development in the adult DG (legend next page)

Figure 1-7 Time course of neuronal development in the adult DG

Progression of neuronal development from 0 days (RGL division) to functional integration of a mature granule neuron (28 days). After the IPC stage, the dendritic tree starts to be built, being mainly formed by day 14. It is also during this time that the first GABAergic excitatory input appears. Glutamatergic input is only detected after this. At 28 days, newly born cells show mature features, with spiny dendrites reaching the molecular layer, an axonal projection extending towards CA3, and with the appearance of glutamatergic inputs and GABAergic inhibitory inputs (see text for more details). (Figure reproduced with permission from Li et al., 2009b)

1.3.4 Regulation of adult neurogenesis in the hippocampus

One of the main characteristics of neural precursors in the hippocampus, and probably one of the main reasons that makes neurogenesis in the adult DG such an intriguing and captivating topic is their being regulated by an ever-increasing number of physiological and pathological stimuli. Importantly, many of the stimuli that have been shown to influence neurogenesis in the hippocampus, have also been shown to cause a corresponding change in cognitive performance (Table 1-1; Li et al., 2009b), emphasising its influence in every-day behaviour and therefore giving the matter even more significance.

Now, what does regulation actually refer to when talking about regulation of adult neurogenesis? And how is regulation different from control in this context? To answer these questions it is useful to go back in time and look at neuronal development in an embryonic context. During embryonic development, when describing the intrinsic and specific genetic programmes that determine the fate of a cell at a specific time, we refer to the *control* of neurogenesis. During adulthood, these genetic programmes are still in place, but here, as an obvious confirmation of the interaction of the hippocampal neurogenic niche with the outer world, it is instead the *regulation* by extrinsic factors that plays a bigger part in the formation of new neurons (Kempermann, 2011b). This distinction between control and regulation, even though blurred at times, makes adult neurogenesis a very unique process, where development is not the final goal, but plasticity and adaptation are. Only the fine-tuned balance between intrinsic control and extrinsic regulation, as well as the careful and relevant integration of these inputs, allows for the correct response to take place and for, in the last instance, the normal function of hippocampal circuits (Kempermann, 2011b).

Another important point to bring forward at this stage is the idea that regulation can occur at many different levels during neuronal development. Not only precursor proliferation can be the target of regulatory mechanisms, but also neuronal survival or integration of new neurons into pre-existing circuits can be affected in response to certain stimuli (Figure 1-8; Aimone et al., 2014). This inevitably adds a further level of complexity to an already complex system.

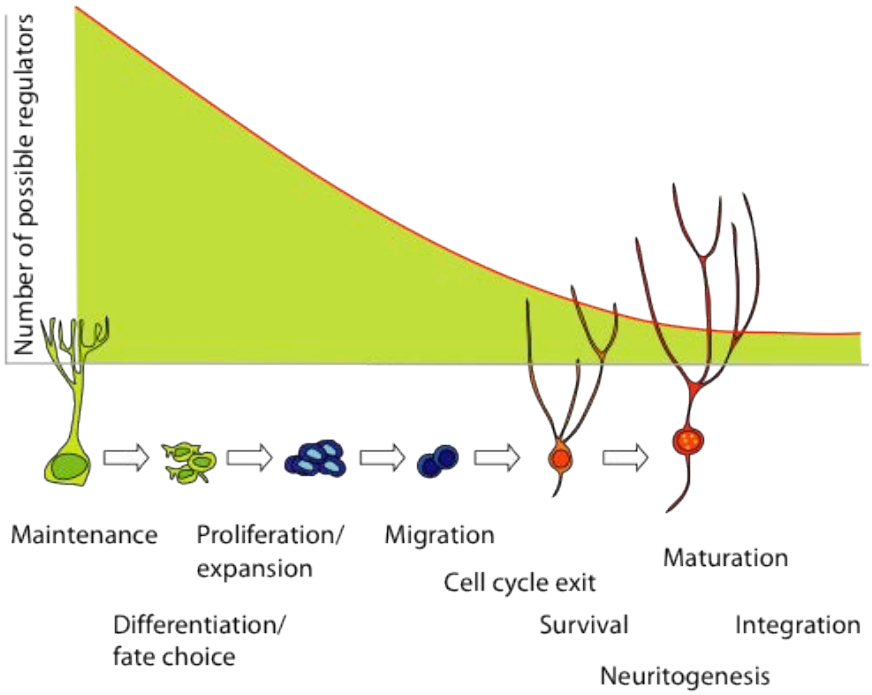

Figure 1-8 Regulatory events in the neurogenic lineage

Neurogenesis in the hippocampus can be regulated at multiple stages during the course of neuronal development, and the possible outcomes of this regulation are limited by decisions made at previous stages. Each step from stem cell maintenance, to IPC proliferation and expansion, neuroblast migration, and neuronal maturation are subjected to external regulation. (Figure reproduced with permission from Kempermann, 2011b)

Here I do not aim to extensively describe each of the stimuli that have been shown to regulate neurogenesis (see Table 1-1 for a list of stimuli that influence adult neurogenesis), but only to briefly illustrate the concept of regulation and mention those environmental factors that will be relevant for this work.

Table 1-1 Environmental stimuli known to regulate hippocampal neurogenesis

Regulator	Effects on neurogenesis	Effects on cognition	References
Physical activity			
Voluntary running	Increased proliferation	Improved learning and memory	van Praag et al., 1999
Enrichment	Increased neuronal survival	Improved learning and memory	Kempermann et al., 1997 Young et al., 1999 Kronenberg et al., 2003
Learning	Increased and decreased neuronal survival (depending on stage of neurogenesis)	Improved learning and memory	Dobrossy et al., 2003
Sleep			
Acute sleep deprivation	Increased proliferation and neuronal survival	NA	Grassi Zucconi et al., 2006
Chronic sleep deprivation	Decreased proliferation	NA	Mirescu et al., 2006
Stress			
Physical stress	Decreased proliferation	NA	Malberg and Dunman, 2003
Psychosocial stress	Decreased proliferation	Impaired contextual memory	Gould et al., 1998 Dong et al., 2004
Ageing	Decreased proliferation	Correlated impairment in learning and memory	Kuhn et al., 1996 Driscoll et al., 2006

The first reports of environmental factors as positive regulators of adult neurogenesis came from experiments showing that mice that lived in an enriched environment had more new neurons in the DG than littermates living in standard cages (Kempermann et al., 1997). It is now generally believed that environmental enrichment is predominantly a survival-promoting stimulus, and that it acts mainly on new-born neurons (Kronenberg et al., 2003, Young et al., 1999), although it can also promote survival of precursors in long-term experiments (Kempermann and

Gage, 1999). There are, however, reports that show different responses to environmental enrichment in different strains of mice (Kempermann et al., 1998a), highlighting the importance of genetic factors in these responses. It is voluntary running that has mainly a positive effect on precursor cell divisions (van Praag et al., 1999), in particular in the proliferation of type 2 IPCs (Kronenberg et al., 2003, Steiner et al., 2008). Interestingly, the effects of these two pro-neurogenic stimuli, which mainly act through different mechanisms, have been shown to be additive, as physical activity together with an enriched environment exerted a larger effect on adult neurogenesis than any one stimulus alone (Fabel et al., 2009).

A number of factors have been linked to environmental enrichment and physical activity and are thought to, at least in part, mediate their survival and proliferative effects. One of these is vascular endothelial growth factor (VEGF). VEGF was first identified as an angiogenic protein, but it is now known to also promote neurogenesis by acting on progenitor cells to increase their proliferation. VEGF is expressed by granule cells of the hippocampus (Lee et al., 2009), while VEGF receptor 2 (VEGFR2 or Flk1) is present on DCX-expressing cells (Jin et al., 2002), a pattern of expression that would correspond with the suggested function. Indeed, blockade of peripheral VEGF abolished the induction of neurogenesis in response to physical activity (Fabel et al., 2003). Similar results were shown for the action of Insulin-Like Growth Factor-1 (IGF-1) in mediating some of the effects of the exercise-induced neurogenic response in rats. This systemically-acting factor was shown to accumulate in neurons during physical activity (Trejo et al., 2001).

Another factor that has been extensively studied with a focus on adult neurogenesis is the brain-derived neurotrophic factor (BDNF). Here, BDNF is regarded as a survival and maturation factor. Chronic infusion of BDNF in the adult DG of rats resulted in an increase of the number of newly born granule cells (Scharfman et al., 2005). Moreover, both physical activity and environmental enrichment lead to increased BDNF expression (Zajac et al., 2010), and lower BDNF levels in heterozygous BDNF knockout mice or impaired TrkB neurotrophin receptor activation, are able to abolish the survival-promoting effect of an enriched environment (Rossi et al., 2006, Sairanen et al., 2005).

Both growth factors, like VEGF and IGF-1, and neurotrophic factors, like BDNF, share a common signal transduction mechanism: ligand binding to one of the tyrosine kinase family of receptors results in its autophosphorylation and later activation of downstream signalling pathways, of which the PI3K/Akt and the MAPK/Erk pathways stand out. IGF-1's proliferative effect on adult hippocampal progenitors has been shown to be dependent on the activation of the MAPK pathway *in vitro* (Aberg et al., 2003). Another study showed inhibition of the PI-3K/Akt signalling pathway by infusion of the inhibitor LY294002 to abolish the increased exercise-induced survival of newly generated neurons, but had no effect on the upregulation of precursor proliferation (Bruel-Jungerman et al., 2009). These results suggest that different trophic factors might have divergent functions by activating different signalling pathways.

In addition to these positive regulators of adult neurogenesis, namely physical activity and environmental enrichment, there are a number of factors that appear to decrease neurogenesis either by directly reducing the number of proliferating precursors and/or by decreasing the number of functional integrating neurons. One of the most studied of these is stress. Stress, both acute and chronic, regulates neurogenesis by decreasing the proliferation of progenitor cells in the DG (reviewed in Mirescu and Gould, 2006). This effect appears to be common among species and stressors, including both physical (e.g. foot shock; Malberg and Duman, 2003), and psychological stressors (e.g. resident-intruder stress and isolation; Dong et al., 2004, Gould et al., 1998). The effects of stress on the production of new neurons are still being investigated, with studies showing contradictory results; some show a suppression of neuron generation and others show periods of enhanced cell survival that follow the decrease in precursor divisions (Mirescu and Gould, 2006).

Even though the understanding of the signalling pathways involved in the anti-neurogenic response following stress is far from clear, substantial evidence now suggests that hormones play an important role in this process. An elevation of glucocorticoid levels is thought to be one of the main mechanisms involved in the stress-mediated suppression of proliferation, since the removal of circulating adrenal steroids by adrenalectomy increased neurogenesis, and the increase of exogenous corticosterone had the opposite effect (Cameron and Gould, 1994).

While a number of physiological stimuli appear to influence the activity of cell types late in the lineage (i.e. type 2, type 3), few of these have been shown to regulate the activity of type 1 RGLs. Whether this is due to a lack of stem cell markers that would allow for a direct investigation of their responses, or whether it reflects a real propensity of stem cells to be partly excluded from external stimulation to avoid their exhaustion, is something it is not known. So far, only epileptic seizures are known to alter RGL's activity. Seizures result from synchronized hyperactivity of excitatory synapses, and they robustly increase precursor cell proliferation (including type 1, type 2 and type 3 cells; Steiner et al., 2008, Parent et al., 1997). Contrary to what would be expected from an increase in proliferation, and possibly due to damage to the neurogenic permissiveness in the niche, the survival of newly generated neurons after chronic or severe seizures is reduced (Mohapel et al., 2004).

1.3.4.1 Adult hippocampal neurogenesis and ageing

The generation of new neurons in the adult DG sees a progressive reduction with increasing age, with a peak during early adulthood and a quick decrease thereafter. This reduction has been shown to be due to a decline in precursor cell proliferation (Kuhn et al., 1996, Kempermann et al., 1998b). I have included this negative regulator of neurogenesis separate from the rest because age can be considered special in a number of ways. Ageing is unavoidable and a fundamental determinant of life, and the reduction of neurogenesis in old age could be seen as a by-product of other age-related systemic and structural changes. Ageing, therefore, might be considered as a default regulatory factor to take into account when studying baseline levels of adult neurogenesis (Klempin and Kempermann, 2007). What is interesting in an ageing context is that physiological regulation of the baseline is still in place, so that both environmental enrichment and physical activity are able to stimulate adult neurogenesis to increase the number of new neurons produced (Kempermann et al., 1998b, van Praag et al., 2005, Kempermann et al., 2002). Moreover, it seems that even though the baseline level in old age is lower, the relative regulation that is possible is larger, as it was shown that the effect of enrichment was, in relative terms, stronger in old animals compared to young ones (Kempermann et al., 1998b).

Despite a general agreement on the idea of loss of neurogenesis with age, the nature of the age-associated decline is still controversial. In the recent model proposed by Encinas and colleagues (2011), where the quiescent stem cell population suffers an activation-dependent deforestation, NSCs lose their stem cell properties and differentiate into astrocytes, making the reduced number of stem cells accountable for the reduced neurogenesis during ageing. This "disposable stem cell" model, as it became known, proposes that, under normal conditions, stem cells are used only once in the adult life; their activation is followed by a series of asymmetric divisions that eventually leads to their final differentiation into an astrocyte (Encinas and Sierra, 2012).

Another model of the force driving age-related neurogenesis decline proposes, instead, that NSCs shift into a predominantly quiescent state, and thus preserve the potential to be activated by external stimuli (Bonaguidi et al., 2011, Bouab et al., 2011, Lugert et al., 2010, Hattiangady and Shetty, 2008). In this case, both a loss of neurogenic signals in the environment or a reduced potential of stem cells to respond to normal signalling could account for the decline in neurogenesis. A number of growth factors, like VEGF, FGF2 and IGF-1, have indeed been shown to decline with age (Shetty et al., 2005); stem cells, nonetheless, appear to maintain the capacity to respond to the neurogenic effects of growth factors. Restoration of IGF-1 levels by intracerebroventricular infusion in aged rats, restored neurogenesis in the hippocampus (Lichtenwalner et al., 2001).

It is likely that both mechanisms are in place in the adult DG, and that their relative contribution depends on other factors acting on stem cells throughout the animals' lifespan. Elucidating the mechanisms underlying age-dependent impairment of neurogenesis and its ultimate impact on hippocampal function will be of great use in understanding the deleterious effects of ageing.

1.3.5 Functional significance of adult hippocampal neurogenesis

The primary function that has mostly been attributed to the adult DG is that of being responsible for the pattern separation of cortical inputs to the hippocampus. Despite extensive characterisation, however, the exact functional significance of newly

formed neurons still remains unclear. The addition of new neurons to an otherwise stable circuit represents quite a unique challenge, especially since we know that the hippocampal neuronal networks undergo such dynamic regulation. Nonetheless, theoretical approaches using computational modelling have suggested that the presence, at any given time, of neurons at different stages of maturation, with each of them presenting distinct properties, could be an advantage, since their contribution at each of these stages could be different from that of mature granule cells (Aimone et al., 2010, Aimone et al., 2014, Marin-Burgin and Schinder, 2012).

A few major hypotheses are being considered when searching for the function of neurons born in the adult hippocampus. Importantly, these do not need to function exclusively from one another. The first couple of these hypotheses are related to pattern separation. Pattern separation is the distinct representation of very similar inputs: two different situations can be composed of many of the same objects or spatial features, yet they might still be stored as distinct non-overlapping memories. Several behavioural studies in mice and rats that performed spatial discrimination tasks, including radial-arm maze and fear context discrimination tasks, have validated this hypothesis (Clelland et al., 2009, Sahay et al., 2011). Related to this idea, is that of immature neurons being important for enhancing pattern separation for events separated in time, and therefore function as pattern integrators of temporally adjacent events (Aimone et al., 2010). These functions would be able to be carried out, as mentioned above, thanks to neuronal activity in the hippocampus being able to undergo differential decoding by a heterogeneous population of hippocampal granule neurons born at different times. Immature neurons have a low activation threshold and are, therefore, highly responsive and integrative, allowing them to encode memories derived from very similar stimuli (Clelland et al., 2009, Marin-Burgin et al., 2012).

More recently, a new and highly controversial hypothesis has been postulated to explain the function of new-born neurons in the adult DG. Based on computational models that predict that neurogenesis during adulthood would lead to the degradation of already established memories, as well as observations that infantile amnesia (or infantile forgetting) is correlated with high levels of hippocampal neurogenesis, a group led by Paul Frankland hypothesised that DG neurogenesis in the postnatal and

adult brains can modulate changes in memory persistence (Akers et al., 2014, Frankland et al., 2013). Using a contextual fear conditioning behavioural paradigm, this group was able to show that increasing neurogenesis after memory formation is sufficient to induce forgetting in adult mice, while decreasing the generation of new neurons, again after the memory has formed, has the opposite effect. These surprising results would suggest that neurogenesis in adult brains promotes the degradation of hippocampus-dependent memories. Further studies will have to confirm these findings and try to reconcile them with other studies in the field.

1.3.6 Adult neurogenesis and neurological disorders

Interest in adult neurogenesis was heightened not only by the recognition that new neurons play specific roles during physiological brain function, but also by reports that suggested an association between arrested or altered neurogenesis and rodent models of both neuropsychiatric and neurodegenerative diseases. In this context, however, it became difficult to establish what is the cause and what the consequence: does pathology affect neuronal precursors and their ability to generate new neurons, or is it a failure of the neurogenic process that might contribute to disease progression? In an expansion of the latter idea, it has been proposed that neurodegeneration, rather than an increase in degeneration, could be the consequence of a lack of plasticity and regeneration, and thus reduced levels of neurogenesis could be underlying a pathogenic process (Steiner et al., 2006). Even though such idea probably only holds true for very specific aspects of disease, it sets the ground to explore the evidence for and against it.

Much of the evidence comes from studies of major depression, where it has been suggested that impaired adult neurogenesis might contribute to the development of the disorder (Jacobs et al., 2000). In models of stress and depression in mice, reduced neurogenesis appears to be one of the prominent features. Moreover, all known antidepressants enhance the number of neurons born in the adult DG, while some of those actually require neurogenesis here to be effective. In humans, patients with chronic depression show a loss of hippocampal volume as well as impairments in hippocampal-dependent functions (reviewed in Jacobs et al., 2000, Sahay and Hen, 2007). Similar findings have been presented for schizophrenia, a severe chronic

mental disorder with complex cognitive and affective symptoms; and these have also led to an adult neurogenesis theory of the disease. Schizophrenic patients have a reduced hippocampal volume, and post-mortem samples show decreased cell proliferation in the DG (reviewed in Reif et al., 2007).

Links between impaired adult neurogenesis and a number of neurodegenerative diseases have also been found. Evidence appears inconsistent at times with both positive and negative modulation of neurogenesis reported. Such inconsistencies can be reconciled if acute and chronic states are distinguished: while acute pathology tends to cause a transient increase of proliferation, a lasting reduction of adult neurogenesis is in general seen after chronic damage (Steiner et al., 2006). In Alzheimer's disease (AD), for example, it has been noted that, during the course of the disease, post-mitotic neurons in the hippocampus are induced to enter the cell cycle, and this attempt normally fails and leads to their death (Nagy, 2000). Of interest is that AD pathology, with β-amyloid (Aβ) plaque accumulation, begins in close proximity to precursor cells in the hippocampus, and presence of Aβ peptide in neuronal progenitor cell cultures has been shown to disrupt neuronal differentiation (Haughey et al., 2002).

Associations to adult neurogenesis also exist with Parkinson disease (PD), which is characterised by dopaminergic cell loss and intracellular deposition of α-synuclein, and Huntington's disease (HD), an autosomal-dominant disorder that leads to striatal degeneration. Data from post-mortem PD samples showed reduced numbers of precursor cells in the DG, a result that was also seen after dopaminergic deafferentiation to the DG in mice (Hoglinger et al., 2004). As for HD, while it had already been recognised that animal models of the disease show reductions in hippocampal neurogenesis, a recent report that showed that new neurons integrate into the striatum throughout life in humans, also showed that these adult-generated striatal neurons are preferentially depleted in HD patients (Ernst et al., 2014). Altogether, these findings open up the doors to an emerging potential to use endogenous neuronal precursors for clinically relevant therapies.

1.4 Mechanisms involved in the control of adult hippocampal neurogenesis

In contrast to the highly proliferative capacity of NSCs in the embryonic nervous system, NSCs in the adult hippocampus exist primarily in a quiescent state. And, unlike senescent or terminally differentiated cells, quiescent cells are able to re-enter the cell cycle and selectively respond to changing physiological demands as well as to aberrant pathological states (Bonaguidi et al., 2011, Encinas et al., 2011, Lugert et al., 2010). Here stem cells are required to continuously receive and integrate external inputs to generate an appropriate response, which for them it will primarily mean: to exit the cell cycle and remain in a dormant or quiescent state, to proliferate and promote neurogenic lineage progression, or to terminally differentiate (Figure 1-9; Beukelaers et al., 2012, Suh et al., 2009). It is the signals received from the niche, together with the mechanisms that intrinsically control the self-renewal and maintenance of the stem cell state that are critical for the correct response to stimuli and for preventing the exhaustion of the progenitor population during adulthood (Morrison and Spradling, 2008, Nakada et al., 2011, He et al., 2009, Schwarz et al., 2012).

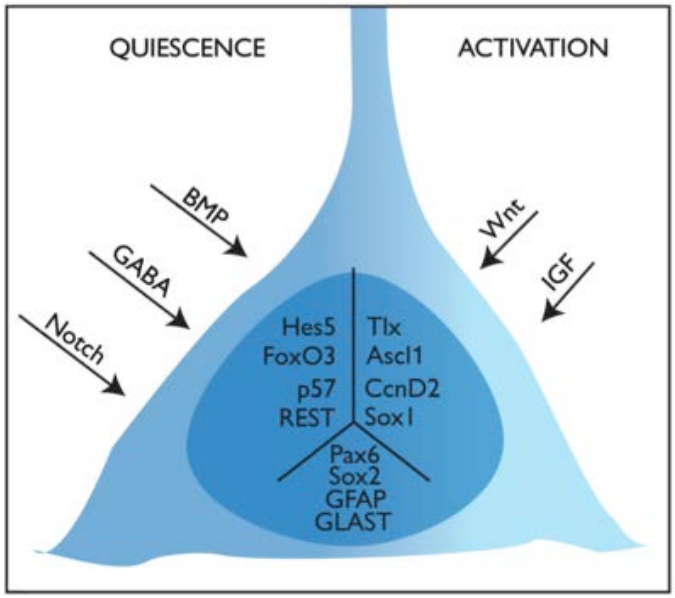

Figure 1-9 The balance between quiescence and activation of stem cells is controlled by a number of extrinsic and intrinsic factors

This scheme shows an RGL and the different extrinsic signals and intrinsic factors known to regulate their activity. The challenge for the future is to understand how all these signals are interpreted and integrated by RGLs (see text for details). (Figure reproduced with permission from Urbán and Guillemot, 2014)

In this section I will describe the mechanisms that are responsible for controlling that the tight balance between stem cell quiescence and self-renewal is under place. Despite all mechanisms working towards the same goal of normal adult hippocampal function and tissue homeostasis, I have divided the text into those mechanisms involved in the maintenance of quiescence, and those important for stem cell proliferation. These are themselves subdivided into extrinsic and intrinsic factors.

1.4.1 Mechanisms involved in the maintenance of quiescence

1.4.1.1 Extrinsic factors

A common mechanism to prevent exhaustion of the progenitor pool is the active suppression of proliferation. For example, IPCs in the SGZ, through Notch ligands in their surface, activate Notch signalling in type 1 stem cells and like this provide a negative feedback regulatory mechanism that maintains the balance between dormant stem cells and activated progenitors. Indeed, conditional ablation of Notch1 or of a downstream effector of Notch activity, RBPJ-κ, in type 1 NSCs resulted in a transient and rapid activation of progenitor proliferation, accompanied by a later depletion of the stem cell pool (Ables et al., 2010, Ehm et al., 2010, Imayoshi et al., 2010). Interestingly, Ehm et al. (2010) found RBPJ-κ to be acting through the transcription factor Sox2 to control stem cell maintenance, which is in accordance with results showing that conditional deletion of Sox2 leads to a loss of RGLs and neurogenesis (Favaro et al., 2009).

Similar to Notch1, BMP signalling was also shown to be essential in regulating the equilibrium between maintenance and proliferation of stem cells. BMPR-1A is expressed by adult hippocampal NSCs, and blockade or inactivation of the BMP pathway in the hippocampus results in an initial stimulation of proliferation and thus an increase in new-born neurons followed by an exhaustion of the stem cell population (Mira et al., 2010). Following this study, Guo et al. (2011) uncovered a mechanism by which BMP signalling is modulated in the DG. Loss of the RNA-binding protein fragile X relative protein 2 (FXR2) resulted in enhanced BMP signalling and therefore increased stem cell proliferation and neurogenesis. FXR2 is

expressed by type 1 DG stem cells, where it colocalises and binds to noggin mRNA. Binding of FXR2 to noggin, a BMP antagonist, leads to reduced mRNA stability and therefore upregulated BMP signalling.

The role of neurotransmitters in controlling stem cell fate has gained momentum in recent years. In particular, the role that GABAergic transmission exerts during adult neurogenesis has received special attention (reviewed in Pallotto and Deprez, 2014). A number of studies have investigated the role of GABA signalling in the adult DG, and all of them are in agreement that GABA action on RGLs is important for promoting their quiescence in response to neuronal activity. Song et al. (2012) provided evidence for a role of γ_2-subunit-containing $GABA_A$ receptors in dictating the stem cells' choice between quiescence and activation. GABA is released by parvalbumin-expressing interneurons found in close proximity to RGLs in the SGZ. Similarly, deletion of the α4-containing $GABA_A$ receptor showed increases in precursor proliferation in the DG of adult mice (Duveau et al., 2011). Giachino et al. (2014), on their part, found the metabotropic $GABA_B$ receptors to respond to GABA to inhibit neurogenesis, although it is yet to be confirmed whether this effect is direct on type 1 cells.

1.4.1.2 Intrinsic factors

A number of transcription factors have been proposed to mediate the activity of these extrinsic signals by inducing quiescence-promoting transcriptional programmes. One of these is FoxO3, a member of a gene family known to extend lifespan in invertebrates. FoxO transcription factors are regulated by Akt-dependent phosphorylation in response to growth factor and insulin stimulation. Phosphorylation of FoxO factors promotes their nuclear export, and thus represses their transcriptional function (Salih and Brunet, 2008). Similar to the phenotypes of Notch and BMP conditional deletions, constitutive ablation of FoxO3 leads to a depletion of the NSC pool in adult mice. This transcription factor is thought to prevent premature exhaustion of NSCs by regulating genes controlling cell cycle re-entry, differentiation, and oxygen and glucose metabolism (Renault et al., 2009). Another of such factors is the zinc finger repressor protein REST, which, by restraining the neurogenic programme, it maintains adult NSCs in a quiescent state;

and this is shown by the depletion of the progenitor pool and the decrease in granule neurons after conditional deletion of REST in SGZ NSCs (Gao et al., 2011). In the postnatal brain, the transcription factor nuclear factor 1/X (NFIX) has also been shown to control the quiescent state of RGLs (Martynoga et al., 2013). NFIX was identified using epigenomic profiling to establish enhancers associated with the quiescent state in BMP4-treated NSC cultures, which, also in this study, were demonstrated to acquire characteristic features of quiescent cells. NFIX gain and loss of function analyses in quiescent NSCs, as well as analysis of NFIX mutant mice suggested that this transcription factor might be controlling, among other aspects of the quiescent phenotype, some important cell adhesion properties, and thus the interaction of NSCs with their environment.

While regulation of transcription is undeniably important in the intrinsic control of stem cell fate, mechanisms acting outside of the transcriptional level have also been identified. The RNA-binding protein fragile X mental retardation protein (FMRP), another member of the family of fragile X mental retardation proteins, was shown to bind to, and inhibit the translation of, Ccnd1, CDK4 and GSK3β mRNAs. Loss of FMRP resulted in increased stem cell proliferation, through Ccnd1 and CDK4 expression, as well as increased astrocyte differentiation at the expense of neuronal differentiation, through downregulation of Wnt signalling and Neurog1 expression induced by increased GSK3β-dependent β-catenin inhibition (Luo et al., 2010).

1.4.2 Mechanisms involved in stem cell and progenitor proliferation

1.4.2.1 Extrinsic factors

One of the most studied extrinsic regulators that has been associated with precursor proliferation in the adult hippocampus is Wnt. The canonical Wnt/β-catenin signalling pathway plays important roles during development, and, notably, it is fundamental for the formation of the hippocampus. Several Wnt proteins, as well as multiple BMPs, are secreted by the CH in the dorsal telencephalon, the hippocampal organizer (Lee et al., 2000). In the adult brain, Wnt signalling has been shown to be required for different aspects of hippocampal neurogenesis, from precursor

proliferation, to neuronal differentiation and neurite maturation (reviewed in Varela-Nallar and Inestrosa, 2013). The first indication pointing at a role in proliferation came from a study showing that inhibition of Wnt signalling almost completely abolished neurogenesis *in vivo*, while overexpression of Wnt3a was sufficient to promote precursor cell activity (Lie et al., 2005). Further studies showed, consistent with this idea, that the blockade of neurogenesis observed after Wnt inhibition resulted in hippocampus-dependent learning deficits (Jessberger et al., 2009, Mao et al., 2009). In embryonic hippocampal progenitor cell cultures, Wnt3a promoted cell proliferation by shortening the duration of the cell cycle (Yoshinaga et al., 2010). It has previously been proposed that the length of G_1 can directly influence the cellular output of neural progenitor cells, and control the balance between proliferative and neurogenic divisions. This "cell cycle length hypothesis", as it became known, indicates that shortening the cell cycle of NSCs during development is sufficient to increase their proliferative potential (Salomoni and Calegari, 2010). This idea was later expanded to also include stem cells in the adult brain. Overexpression of Cdk4 and Ccnd1 in the hippocampus, two factors essential for G_1 progression, resulted in a shorter cell cycle and an expansion of the NSC pool at the expense of neurogenesis (Artegiani et al., 2011). Keeping this in mind, Yoshinaga et al. (2010), suggest that Wnt in the developing hippocampus could be acting by regulating Ccnd1 expression, a well-known Wnt target, to shorten the cell cycle and promote proliferation. Further studies will be required to establish whether this might also be true for the adult brain.

Wnt signalling in the adult hippocampus has also been implicated in the regulation of neurogenesis in a number of physiological and pathological conditions, and ageing is a good example of this. Secretion of Wnt3a by astrocytes was shown to reduce with age, and to act to decrease hippocampal neurogenesis (Miranda et al., 2012, Okamoto et al., 2011). Interestingly, however, this decline was found to be reversible. Exercise was able to restore the expression levels of Wnt3a and thereby rescue impaired neurogenesis in aged animals (Okamoto et al., 2011). Modulation of Wnt signalling with inhibitors secreted in the niche is also an important mechanism involved in the age-dependent reduction of neurogenesis. The expression of the inhibitor dickkopf-related protein 1 (Dkk1), for example, was reported to increase

with age to negatively modulate neurogenesis (Seib et al., 2013). A similar mechanism was proposed for the observed activity-dependent increase in precursor proliferation. Neuronal activity is proposed to decrease the expression of the granule neuron-produced Wnt inhibitor secreted frizzled-related protein 3 (sFRP3) to, in this case, positively regulate neurogenesis (Jang et al., 2013).

Despite the undisputed importance of Wnt in regulating neurogenesis in the adult brain, some questions still remain. For example, it will be important to ascertain specifically which cells respond to Wnt, as well as to discriminate Wnt action on stem cells versus progenitors. A study looking at NSCs in the SVZ, for instance, has reported Wnt signalling only to be upregulated in stem cells dividing symmetrically, as it is the case in response to stroke or regeneration, but to be absent in stem cells dividing asymmetrically (Piccin and Morshead, 2011).

Another extrinsic factor that has been studied and was shown to be important for precursor proliferation in the context of adult neurogenesis is Shh. Enhancement of Shh signalling with an agonist resulted in increased proliferation in both the DG and SVZ (Machold et al., 2003). What the sources of Shh are in the hippocampus is still unclear. However, both quiescent RGLs and IPCs were shown to respond to Shh, and these Shh-responsive cells were shown to contribute to on-going neurogenesis (Ahn and Joyner, 2005). Moreover, RGLs in the DG possess primary cilia. Conditional ablation of these primary cilia resulted in a reduction in the number of IPCs (Amador-Arjona et al., 2011), corroborating the idea that Shh signalling is important for precursor proliferation.

1.4.2.2 Intrinsic factors

The activation of NSCs requires a switch in the transcription factor programme, so that quiescent type 1 cells re-enter the cell cycle and promote lineage progression. The nature of this switch is currently not known, but a few clues are starting to emerge. Recent work described the nuclear receptor TLX to control NSC activation in postnatal mice, and thus ensure the proliferative ability of these cells, as well as to contribute to spatial learning and memory circuits. TLX loss leads to non-proliferative NSCs that can be re-activated upon TLX reintroduction (Niu et al.,

2011, Zhang et al., 2008). A number of factors have been proposed to collaborate with TLX to maintain stem cell self-renewal. For example, recruitment of histone deacetylases (HDACs) by TLX to target genes like p21 and PTEN, which normally supress proliferation, was reported to repress gene expression to maintain RGLs' in an undifferentiated and proliferative state (Sun et al., 2007). Similarly, TLX cooperates with HDACs to inhibit transcription of microRNA 9 (miR-9) in proliferating cells, and thus supress neuronal differentiation (Zhao et al., 2009). In the SVZ, TLX was also shown to activate Wnt/β-catenin signalling, contributing to the stimulation of precursor proliferation (Qu et al., 2010)

Different factors are involved in the maintenance of proliferation of the intermediate population of progenitors. This stage in the neurogenic process is marked by the up-regulation of the transcription factors Neurog2 and Tbr2. Absence of Neurog2 resulted in a reduction of hippocampal granule neurons of juvenile animals, suggesting a role for Neurog2 on the maintenance of the progenitor state and thus for the lineage amplification that takes place during this stage (Roybon et al., 2009). In the case of the transcription factor Tbr2, its conditional deletion leads to a depletion of both IPCs and neuroblasts, also implicating Tbr2 in the generation and maintenance of late progenitors in the adult SGZ (Hodge et al., 2012). Here, progression of the lineage into IPCs is proposed to take place by Tbr2-mediated repression of Sox2.

Interestingly, the loss of type 2 proliferating progenitors in the Tbr2 mutant impacts on the type 1 population. Tbr2 is suggested to exert a negative feedback onto the NSC pool (Hodge et al., 2012). Such non-cell autonomous regulatory feedback mechanism controlling NSC proliferation has also been shown for Prox1 and Jagged1 mutants, where conditional inactivation of these genes leads to a transient expansion and later exhaustion of the NSC pool. This process has been proposed to be mediated by the loss of active Notch signalling in adult NSCs, which, in turn, is a consequence of an absence of IPCs, the Notch ligand-expressing cells in the hippocampal DG (Lavado et al., 2010, Lavado and Oliver, 2014). This mechanism is also important as an example of the link between stem cell maintenance and the generation of appropriate numbers of progeny in the SGZ.

1.5 The proneural factor Ascl1 in neurogenesis

1.5.1 Proneural factors and their mechanism of action

During embryonic brain development combinations of transcription factors control the generation of cell diversity in the nervous system in a spatio-temporal manner (Guillemot, 2007). Proneural proteins are key regulators of this process, coordinating the acquisition of a neuronal fate and promoting the generation of neuronal cell types (reviewed in Bertrand et al., 2002, Ross et al., 2003). The main mouse proneural proteins are Ascl1 (Mash1), neurogenin 1-3 (Neurog1-3) and Math1 or Atoh1 (Figure 1-10).

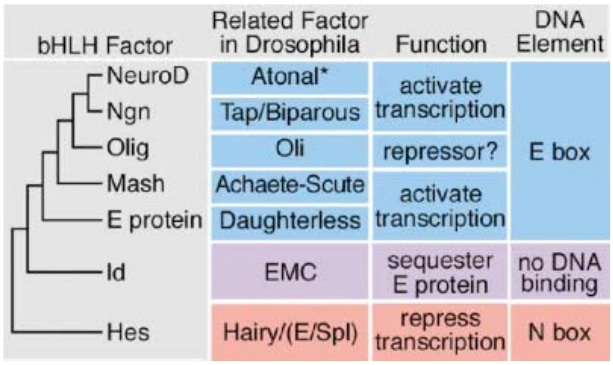

Figure 1-10 bHLH factors in cortical development

Phylogenetic tree showing the relationship between different bHLH factors, and table showing their related factors in *Drosophila*, their function during cortical development and the DNA element they bind to. (Figure reproduced with permission from Ross et al., 2003)

Proneural proteins are basic helix-loop-helix (bHLH) transcription factors. Proteins in this family share a bHLH motif that is evolutionary conserved from yeast to plants and metazoans, including mammas. The common motif is a ≈60 base pairs (bp)-long DNA-binding motif that consists of a basic domain followed by two alpha helices that are connected by a flexible loop (helix-loop-helix domain). The basic region of this domain fits in the main groove of the DNA, where most of the bHLH residues that contact DNA (7 out of 10) are located, while the HLH region is responsible for the dimerization with other bHLH proteins (Figure 1-11; Bertrand et al., 2002).

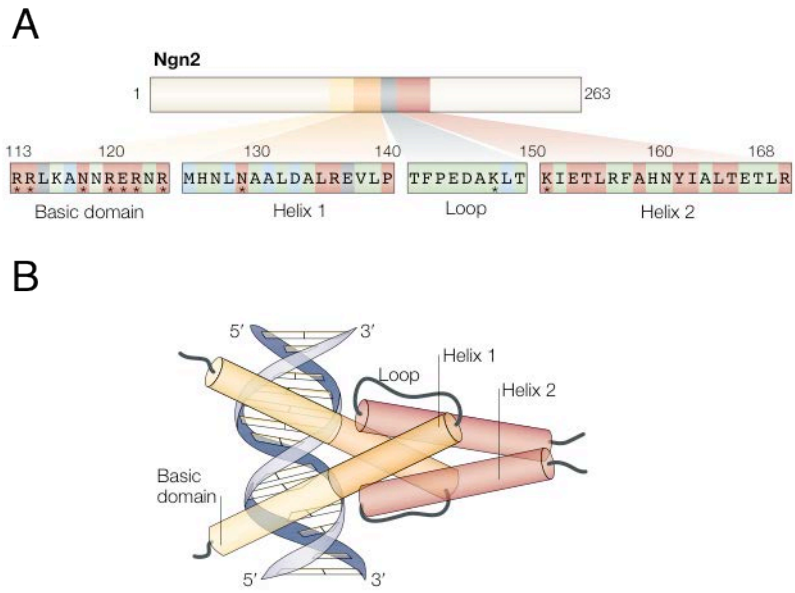

Figure 1-11 Structure of bHLH proteins

(A) Sequence of the bHLH domain of the mouse proneural protein neurogenin 2 (Ngn2 or Neurog2) showing the different regions forming it. The basic domain is followed by two alpha helices connected by a flexible loop. Colour coding indicates the degree of amino-acid conservation between different bHLH proteins (red = residues common to all neural bHLH proteins). Asterisks mark the residues that make direct contact with DNA. **(B)** Representation of the structure of a bHLH dimer complexed to DNA. The basic region fits in the main groove of DNA. (Figure reproduced with permission from Bertrand et al., 2002)

Proneural factors were first discovered as regulators of the early steps of neural development in *Drosophila melanogaster*. In vertebrates, their isolation was possible on the basis of sequence conservation with their fly counterparts (Figure 1-10; Bertrand et al., 2002). As transcription factors, their function is to regulate target gene transcription, and they do so by binding specific hexa-nucleotide sequences of DNA of the type CANNTG called E boxes. They usually do so as homo- or heterodimeric complexes that are formed with ubiquitously expressed E-proteins, including the alternative splice variants of the E2A gene, E12 and E47 (Murre et al., 1989, Johnson et al., 1992). Dimerization is a requisite for DNA binding, and factors interfering with this step will act as repressors of proneural gene activity. Proteins of the Inhibitor of differentiation (Id) family are an example of this; despite having a HLH domain, they lack the basic domain for DNA binding and can, therefore, act as molecular bait to sequester other bHLH factors (Massari and Murre, 2000).

Proneural proteins act mainly as transcriptional activators, with only a few of them, including Olig2, shown to act as repressors (See Figure 1-10). Regulation of target gene expression by transcription factors depends on transcriptional protein complexes, which include both other transcription factors and cofactors. Activation of gene expression, in the case of the Neurog proteins, for example, was shown to be mediated through the recruitment of cofactors such as p300, CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF; Koyano-Nakagawa et al., 1999). Moreover, interactions between different transcription factors are thought to be essential for the specificity towards different target genes at subsequent stages of development or in different cell types. Castro et al. (2006), for example, have shown that the regulation of the gene encoding the Notch ligand, Delta1, involves cooperative binding of Ascl1 and the POU proteins Brn1 and Brn2. These transcription factors bind adjacent motifs on the *Delta1* promoter, and this interaction is required for transcriptional activation. In addition, through a screen for conserved Ascl1-Brn motifs *in silico*, they found additional candidate target genes that are recognised by these factors and that regulate multiple steps of neurogenesis, providing an example of transcription factor interactions coordinating different neurogenic programmes.

An essential role of proneural proteins in the developing brain is to restrict their own activity to single progenitor cells. In this way, they inhibit differentiation of adjacent cells and ensure the generation of the appropriate number of neurons and glia. This process, named "lateral inhibition" is achieved through activation of the Notch signalling pathway. Proneural genes directly activate the transcription of Notch ligands, namely *Delta* and *Jagged*, which will go on to activate the Notch signalling cascade in neighbouring cells, ultimately resulting in the expression of proneural gene repressors like the *Hes* genes (Imayoshi and Kageyama, 2014).

1.5.2 Ascl1 during cortical development

The proneural protein achaete-scute complex homolog-like 1 (Ascl1; also known as Mash1) directly regulates different steps in the neurogenic programme in the embryonic telencephalon. Expression of Ascl1 in this region of the developing brain is observed in both dorsal and ventral domains. While in the dorsal telencephalon,

where progenitors of the cerebral cortex reside, Ascl1 is co-expressed with Neurog1 and Neurog2, Ascl1 is the only known proneural gene to be expressed in the ventral telencephalon (Figure 1-12; Nieto et al., 2001, Britz et al., 2006, Casarosa et al., 1999). In this ventral region Ascl1 plays a critical role in the generation of cortical GABAergic interneurons, which are generated in the medial ganglionic eminence (MGE) to then migrate into the neocortex. In mice null for Ascl1, the MGE is completely absent, while the lateral ganglionic eminence, where striatal projection neurons are produced, is severely reduced in size (Nieto et al., 2001, Fogarty et al., 2007). Moreover, in the absence of neurogenins, Ascl1 is upregulated in the dorsal telencephalon and induces the expression of ventral GABAergic markers (Parras et al., 2002).

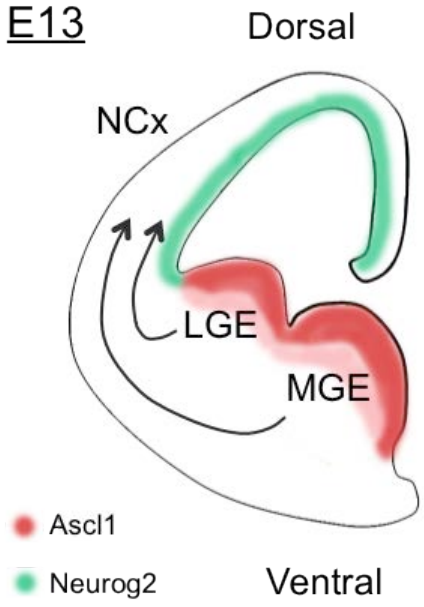

Figure 1-12 Ascl1 and Neurog2 expression in the developing telencephalon

Diagram showing the E13 developing telencephalon. Neurog2 (green) is expressed in the dorsal telencephalon, where the neocortex (NCx) progenitors reside. Ascl1 (red) is expressed in the ventral telencephalon, and here it is important for the generation of GABAergic interneurons. These are generated in the medial ganglionic eminence (MGE) and migrate up into the cortex. Ascl1 also colocalises with Neurog2 in the dorsal telencephalon (not shown). LGE = lateral ganglionic eminence.

A role for Ascl1 in later stages of neuronal development has also been uncovered. Ascl1 promotes neuronal migration in the cortex through regulation of the Rho GTPase Rnd3, and in this way integrates the process of neuronal migration

with other events in the neurogenic programme (Pacary et al., 2013, Pacary et al., 2011, Azzarelli et al., 2014).

Analysis of Ascl1's transcriptional targets in NSC cultures and in the embryonic brain on a genome-wide scale unravelled a novel and unexpected function for this proneural factor. The cellular processes represented among the targets identified suggested Ascl1 to be involved in diverse functions during the neurogenic programme. Some of these included the control of neural progenitor specification, neuronal differentiation and neurite outgrowth, as expected from previous functional studies. However, a large number of genes involved in cell cycle progression were also identified, and loss of function analysis confirmed that Ascl1 is indeed required for normal progenitor divisions (Castro et al., 2011). All together, this data highlights that Ascl1 plays multiple and crucial roles during early and late phases of development of the brain.

1.5.3 Ascl1 in the adult brain

Despite the importance of this factor during embryonic neurogenesis, little was known about the role that Ascl1 played during adulthood. In the DG, it was initially believed that Ascl1 expression corresponded with a transition state between NSCs and IPCs (Lugert et al., 2010, Lugert et al., 2012). New reports, however, demonstrated that Ascl1 was not restricted to early IPCs but also expressed in a subset of radial, type 1 NSCs (Kim et al., 2011, Breunig et al., 2007). Kim and colleagues traced Ascl1 lineage cells in the adult brain using an *Ascl1-CreERT2* mouse line and showed that this factor is expressed in a subset of self-renewing and neuron-generating cells in both neurogenic regions in the adult brain. More specifically, they found that Ascl1 was expressed at low levels in a subpopulation of NSCs in the SGZ and SVZ and that these levels increased as the cells transited to an intermediate progenitor stage. Yet the function of Ascl1 in these cells during neurogenesis remained unknown.

Interest in Ascl1 and its expression in the adult brain has also grown in the past years due to the finding that ectopic expression of this gene can reprogram various cell types into neurons (Berninger et al., 2007, Yang et al., 2011). Moreover,

depending on the presence or absence of other factors, as well as the starting cell type or the time of expression, Ascl1 can induce the generation of several different neuronal fates, from glutamatergic to dopaminergic neurons (reviewed in Arlotta and Berninger, 2014, Yang et al., 2011). In the adult brain, Notch signalling was shown to be reduced in astrocytes after stroke, and this reduction was necessary for striatal astrocytes to enter a neurogenic programme. Blockade of Notch signalling in astrocytes triggered their activation even in the absence of stroke, and this activation was accompanied by Ascl1 expression (Magnusson et al., 2014). Whether Ascl1 expression was required for astrocyte activation was not addressed. However, preliminary results from our collaborator Masato Nakafuku point to this possibility. Ectopic overexpression of Ascl1 using *Glast-CreERT2* mice crossed to *TetO-Ascl1-IRES-EGFP* resulted in the generation of DCX^+ neurons from parenchymal astrocytes, providing them with a neurogenic capacity (Simic, M. and Nakafuku, M., personal communication).

1.6 Aims of the present work

The discovery of the existence of neurogenic niches in the adult brain of mammals, where new neurons are generated throughout life, opened the doors to a new era in the study of the brain. The presence of neural precursors that respond to environmental regulation inspired new research avenues aimed at understanding the molecular mechanisms that control the different steps in the neurogenic programme, as well as how external regulation, of both physiological and pathological nature, is integrated by the precursor pools to maintain tissue homeostasis.

The clinical relevance of these approaches becomes apparent when realising that failure of neurogenesis in the adult brain of both rodents and humans has been associated with a number of neuropsychiatric and neurodegenerative diseases. Understanding how the basic mechanisms underlying neurogenesis can go wrong in the progression of these diseases, might highlight possible treatments to reverse at least some of the symptoms in the pathogenic process. And ultimately, loss of neurons in these neurological diseases might be counteracted by the development of stem-cell-based transplantation therapies, or even by relying on the stimulation and recruitment of endogenous stem cells (Lindvall and Kokaia, 2006).

The adult hippocampus is quite an extraordinary system where new neurons that contribute to behaviour are formed in response to the needs of the organism. Despite knowledge of the mechanisms involved in the control and regulation of this process having grown exponentially in the past couple of years, a number of questions remain. One question that is of particular interest is how stem cells are able to respond to signals from the environment. We know of a number of transcription factors that are important for promoting the proliferation of precursor cells, but how these integrate extrinsic stimuli and how this is translated into a response has not yet been addressed.

The proneural transcription factor Ascl1 plays key functions in the developing brain and appears to also be a potent neurogenic factor *in vitro*. This knowledge, together with the finding that Ascl1 is also expressed in a subset of self-renewing cells in neurogenic regions in the adult brain led me to formulate the hypothesis that Ascl1 is involved in the process of neurogenesis in the adult murine hippocampus. The overall aim of this PhD work is, therefore, to investigate the role that Ascl1 plays in the neurogenic programme in this area of the adult brain. For this overall aim, a number of sub-aims are outlined:

a) Determine where Ascl1 is expressed in the adult hippocampus, i.e. which cells in the neurogenic lineage express Ascl1
b) Study the function of Ascl1 in the specified cells by following a deletion approach, i.e. what is the outcome of deleting Ascl1 in the adult hippocampus
c) Study Ascl1's expression in response to defined neurogenic stimuli
d) Determine the molecular mechanism by which Ascl1 performs its function

Chapter 2 Materials and Methods

2.1 Animals

All procedures were performed in accordance with a UK Home Office Project Licence and approved by the local ethics committee. Mice were housed in standard cages (31cm L, 13.5cm W, 11.5cm H) under a 12 h light/dark cycle, and had *ad libitum* access to food and water. *Glast-CreERT2* mice, which allow for tamoxifen (TAM)-inducible expression of cre recombinase under the astrocyte-specific glutamate aspartate transporter promoter (Glast; Mori et al., 2006), were crossed with *Ascl1*$^{neoflox/neoflox}$ mice (Pacary et al., 2011), in which exon 1 of the *Ascl1* gene is flanked by *loxP* sites, and with *Rosa26-floxed stop-YFP* reporter mice (R26 YFP; Srinivas et al., 2001) to generate *Glast-CreERT2 / Ascl1*^{neoflox}*/ R26 YFP* mice. In order to remove the *PGK promoter-neo* cassette from the *Ascl1* locus, *Ascl1*^{neoflox} animals were crossed with an *actβ-Flp* mouse line (The Jackson Laboratories, Maine). *RBPJk*$^{loxp/loxp}$ brains were kindly provided by Christian Göritz and Jonas Frisén (Department of Cell and Molecular Biology, Karolinska Institute, Sweden) and were generated as previously described (Han et al., 2002). These were crossed with *GLAST::CreERT2* mice (Slezak et al., 2007) to delete *RBPJk* in an astrocyte-dependent manner. *RBPJk*$^{loxp/+}$ animals were used as controls. The *Ascl1*$^{\Delta/\Delta}$ line was generated as described previously (Guillemot et al., 1993). *Nestin-CreERT2* animals (Lagace et al., 2007) were purchased from The Jackson Laboratories (Maine), and crossed with MF1 *wild-type* (*WT*) mice. Both male and female transgenic mice were included in the analyses, unless otherwise stated.

2.2 Genotyping

All animals were genotyped using genomic DNA extracted from ear biopsies. Samples were incubated overnight in 1M Tris pH 8.5, 0.5M EDTA, 10% SDS, 4M NaCl, 100mg/ml proteinase K at 55°C, and DNA was precipitated by centrifugation (13,000 rpm, 10 minutes) with one volume of isopropanol. Genomic DNA was resuspended in 100µl of water. Polymerase chain reaction (PCR) for *Ascl1*^{neoflox}, *Ascl1*^{flox}, *Ascl1 Δ* and *R26 YFP* was performed using REDTaq DNA Polymerase mix (Sigma-Aldrich), following the manufacturer's instructions. PCR for cre was performed using 500mM KCL, 100mM Tris-HCL pH 8.3, 20mM $MgCl_2$, 1.5mM dNTPs, 22.5ng of each reverse and forward primer and 1.25 units of Thermoprime

DNA polymerase (Thermo Scientific ABgene), or GoTaq Green Master Mix (Promega), following the manufacturer's instructions. All PCR programs consisted of: an initialization of 5 minutes at 94°C; a repeated amplification cycle (number of cycles specified below) consisting of a denaturation step of 1 minute at 94°C, an annealing step of 1 minute (temperature specified above), and an extension of 1 minute at 72°C, and a final extension step of 10 minutes at 72°C. The primers used and the PCR temperatures and number of amplification cycles used for each allele genotyped are shown in Table 2-1.

Table 2-1 PCR primers and conditions

Primer	Sequence	Annealing Temp (°C)	Amp cycles
Ascl1 WT *Ascl1^{neoflox}* *Ascl1^{flox}*	5' - CTA CTG TCC AAA CGC AAA GTG G - 3' 3' - GCT CCC ACA ATC CTC GTA AAG A - 5' 3' - TAG ACG TTG TGG CTG TTG TAG T - 5'	64	35
Cre	5' - ATC CGA AAA GAA AAC GTT GA - 3' 3' - ATC CAG GTT ACG GAT ATA GT - 5'	55	28
RYFP	5' - AAA GTC GCT CTG AGT TGT TAT - 3' 5' - AAG ACC GCG AAG AGT TTG TC - 3' 3' - GGA GCG GGA GAA ATG GAT ATG - 5'	58	35
Ascl1 Δ	5' - GCAGCGCATCGCCTTCTATC - 3' 3' - CCAGGACTCAATACGCAGGG - 5'	64	40

2.3 *In vivo* studies:

2.3.1 Tamoxifen and BrdU administration

For activation of the CreERT2 recombinase, postnatal day 60 (P60) animals were administered 4-Hydroxytamoxifen (TAM, Sigma-Aldrich) for five consecutive days (intraperitoneally, i.p.; 2 mg/day, stock solution 10 mg/ml dissolved in 20% EtOH/80% sunflower oil). For mosaic experiments, P60 animals received a single TAM injection at the same concentration (based on preliminary studies with a range of concentrations that showed a single injection to produce adequate mosaic recombination). All *WT* and experimental animals received TAM injections to avoid

injection-related differences. To examine progenitor proliferation, mice were given a single bromodeoxyuridine (BrdU, Sigma-Aldrich) i.p. injection (2 mg, stock solution 10 mg/ml dissolved in 0.9% saline) 2 hours prior tissue collection. In order to examine label-retaining cells a long-term BrdU paradigm was performed. Mice received five daily i.p. BrdU injections (2 mg/day) followed by five consecutive days of BrdU-containing drinking water (1 mg/ml). Mice were sacrificed 20 days after the last day of receiving BrdU in the drinking water.

2.3.2 Voluntary running

To study the activation of stem cells under physiological conditions we used a voluntary running paradigm. Eight to nine week-old female MF1 mice were exposed to a running wheel for 12 days. Two mice were placed per cage, and daily checks were performed to ensure both of them exercised. Control mice were littermates of experimental mice, and they were also housed two per cage. Cages with the running wheel were bigger (34.5cm L, 18cm W, 14cm H) than standard cages for controls (31cm L, 13.5cm W, 11.5cm H).

2.3.3 Social isolation

In order to test the effect of an anti-neurogenic stimulus on stem cell activity we performed a social isolation paradigm. Eight to nine week-old male MF1 mice were initially housed in big cages (45.5cm L, 32.5cm W, 15.5cm H) with 6-8 littermates per cage. These cages also contained chewable toys and at least two red polycarbonate mouse houses. On Day 0 of the experiment animals were placed one per cage in standard cages with no enrichment (31cm L, 13.5cm W, 11.5cm H). Animals were sacrificed one or two days after isolation and processed as described below.

2.3.4 Kainic acid administration

In order to study stem cell activation, we also made use of the activating activity of the seizure-inducing agent kainic acid (KA). Eight to nine week-old MF1 or P86 *Ascl1 WT* and *Ascl1*^{neo}*cKO* male mice received KA (Sigma-Aldrich) as a single i.p. injection at 19 mg/kg dissolved in 0.9% saline (sub-seizure concentration based on

preliminary studies showing that progenitor proliferation is induced without animals undergoing observable convulsions). Animals were monitored for 90 minutes after KA injection, and their behaviour was scored every 10 minutes from 0 to 7 based on Monory et al., (2006) (0- no response, 1- immobility and staring, 2- forelimb and/or tail extension, rigid posture, 3- repetitive movements, 4- rearing and falling, 5- continuous rearing and falling, 6- severe clonic-tonic seizures, 7- death). Animals reaching stage 4 were immediately killed and were not used for the experiment. Around 15% of all animals receiving KA reached stage 4. Remaining animals were then sacrificed 1, 2 or 4 days after KA injection, and processed as described below.

2.4 Expression pattern analysis

2.4.1 RNA probes

The riboprobe used to visualize *Ascl1* mRNA expression was previously described (Guillemot and Joyner, 1993). The riboprobe for Neurog2 was prepared by attaching T7 (5' - TAATACGACTCACTATAGGGAGA - 3') and SP6 (5' - ATTTAGGTGACACTATAGAAGNG - 3') promoters to the probe-specific forward and reverse primers, respectively. E14.5 cDNA from the telencephalon (prepared by Noelia Urbán) was used as template to identify the appropriate sequences with the following primers: forward 5' - GCAACTGGTCCCTGTGATC - 3' and reverse 5' - ATGAAGCAATCCTCCCTCC - 3'. Labelled complementary RNAs were synthesised by *in vitro* transcription. Briefly, sequences amplified by PCR were transcribed in the presence of digoxigenin (DIG)-marked nucleotides (Roche) under standard reaction conditions together with T7 or SP6 RNA polymerases for generation of anti-sense and sense probes, respectively.

2.4.2 Tissue preparation

For immunohistochemistry, animals were anesthetized and transcardially perfused with 0.9% saline for 3 minutes followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 12 minutes. Brains were post-fixed with 4% PFA for 2 hours at 4°C and sectioned coronally at 40μm using a vibratome (Leica). Sections were kept at 4°C on 0.02% azide until used. For p57 immunostaining, brains were cryo-protected in sucrose overnight (30% sucrose in PBS) before being

embedded in gelatin and sucrose (7.5 and 15%, respectively, in PBS) and frozen in isopentane. Frozen brains were cut in a Microm Cryostat (Zeiss) at 30µm and kept at minus 20°C until used. To avoid degradation of the RNA for *in situ* hybridization, the perfusion was performed using diethylpyrocarbonate (DEPC)-treated PBS (1/1,000), and PFA also prepared in DEPC-treated PBS. Dissected brains were cut in half before overnight post-fixation with 4% PFA. Each half was cryo-protected by placing it in 20% sucrose overnight, and then frozen on dry ice in OCT compound (VWR Chemicals) using a plastic mould. Brains were cut in a cryostat (CM3050S, Leica) at 14µm and kept at minus 20°C until used.

2.4.3 Immunohistochemistry

To visualise protein expression in the adult DG, we performed immunohistochemistry. Free-floating sections were blocked in 10% normal donkey serum and 1% Triton X-100 for 2 hours. Sections were then incubated overnight at 4°C with primary antibodies diluted at appropriate concentrations in incubation solution (10% normal donkey serum and 0.1% Triton X-100). See Table 2-2 for a list of primary antibodies and the concentrations they were used at. After 3 washes with PBT (PBS + 0.1% Triton X-100), sections were incubated in incubation solution with corresponding secondary antibodies for 2 hours at a dilution of 1/1000. Secondary antibodies used were conjugated to Alexa-488, Alexa-568 (Invitrogen Life Technologies), Cy3 or Cy5 (The Jackson Laboratory). Following 3 washes with PBT, sections were incubated with 6-diamidino-2-phenylindole (DAPI, 1/10000, Sigma-Aldrich) for 20 minutes to obtain nuclear staining, and finally mounted in Aqua PolyMount (Polysciences).

For staining with rabbit anti-Tbr2, brain sections underwent an antigen retrieval treatment. For this, sections were incubated in sodium citrate buffer (10 mM trisodium citrate, pH 6.0) at 90°C for 20 minutes. Following incubation, sections were left to cool down, rinsed with PBS, and immunostaining procedure was continued as normal. For BrdU immunohistochemistry, staining for other markers was performed first as explained above. Sections were then fixed for 30 minutes with 4% PFA, washed 3 times with PBS and then pre-treated with pre-warmed 2N HCL for 30 minutes. BrdU primary antibody was then added after two 15-minute borate

buffer (0.1 M sodium tetraborate, pH 8.5 in PBS) washes to continue immunostaining as normal.

Table 2-2 Details of the primary antibodies used in this study

Antibody	Species	Concentration used	Manufacturer	Catalogue number
Ascl1	Mouse	1/100	BD Pharmingen	556604
BrdU	Rat	1/1000	AbD Serotec	OBT0030CX
CD31	Rabbit	1/200	Abcam	ab28365
Doublecortin	Goat	1/50	Santa Cruz Biotechnology	sc-8066
GFAP	Rabbit	1/1000	Dako	Z033401
GFAP	Rat	1/1000	Invitrogen Life Technologies	13-0300
GFP	Rabbit	1/1000	Invitrogen Life Technologies	A11122
GFP	Rat	1/1000	Fine Chemicals	04404-84
GFP	Sheep	1/1000	AbD Serotec	4745-1051
Ki67	Rabbit	1/200	Leica	NCL-Ki67p
MCM2	Goat	1/100	Santa Cruz Biotechnology	sc-9839
Nestin	Mouse	1/200	Millipore	MAB353
NeuN	Mouse	1/500	Millipore	MAB377
Olig2	Rabbit	1/200	Millipore	AB9610
p57	Rabbit	1/200	Sigma	P0357
S100β	Rabbit	1/200	Dako	Z0311
Sox2	Goat	1/200	Acris Antibodies	GT15098
Tbr2	Rabbit	1/500	Abcam	ab23345

2.4.4 Microscopic analysis and quantification

The total number of single, double or triple antigen-positive cells was counted in every ninth 40μm section through the entire rostrocaudal length of the DG (-0.82 mm to -4.24 mm from bregma). Images were acquired using an SP5 confocal microscope (Leica). 15 to 20 z-plane images separated by a 1μm step were obtained per section. To present total numbers per dentate gyrus, cells counted were divided by the number of z-planes counted to obtain the number of cells per 1μm, and then multiplied by the total length of the dentate gyrus.

Quantification of antigen-positive RGLs within the total population of RGLs was performed by calculating the percentage of antigen-positive $GFAP^+$ RGLs among the total number of RGLs counted (between 60 and 150). For these counts, cells were deemed to be radial if the cell body, clearly associated with a DAPI-positive nucleus, was located in the SGZ and had a single radial $GFAP^+$ process extending through the GL (for at least two extra $DAPI^+$ nuclei parallel to the SGZ base).

The total number of stem cells in the DG was calculated by counting the number of GFP^+ $GFAP^+$ $Nestin^+$ radial cells within a defined area in z-projections of three z-planes that were 1μm apart. At least two separate areas were quantified per DG, and the same total area was counted per animal.

In each experiment the DG of 3 or more mice per group were used for quantification. In all figures, the cell numbers counted in *WT*, *Ascl1^{neo}cKO* and *Ascl1cKO* mice are numbers of YFP^+ $marker^+$ double-labelled cells, while the numbers counted in *Ascl1^{neoflox}* and *Ascl1^{flox}* mice are for $marker^+$ cells only since YFP is not expressed in these mice.

2.4.5 *In situ* hybridisation

Visualization of mRNA expression on brain sections was carried out by *in situ* hybridisation (ISH). Sections, following rehydration with PBT (0.1% Tween, Sigma-Aldrich, in DEPC-treated PBS), were treated with triethanolamine and 0.25% acetic anhydride to acetylate the amino groups on the proteins and reduce the background.

After a 15-minute pre-hybridisation step with hybridisation solution (50% formamide, 25% SSC, 10% Denhardt's solution, 500 µg/ml herring sperm DNA, 250 µg/ml torula yeast RNA) at room temperature, sections were incubated with the DIG-labelled RNA probes diluted in hybridisation solution (1/100) in a humidified chamber at 70°C overnight. To detect the riboprobes hybridised to the target RNAs, sections were incubated with an anti-DIG antibody conjugated with alkaline phosphatase. To reveal the signal, nitro blue tetrazolium 5-bromo 4-chloro 3 indolyl phosphate (BCIP/NBT, Sigma-Aldrich) was used.

2.5 Fluorescence-activated sell sorting

In order to determine possible Ascl1 target genes playing a role in the adult hippocampus, we sorted stem cells from *Ascl1 WT* and *Ascl1^{neo}cKO* mice. TAM was administered for 5 consecutive days at P60, and DGs were dissected 4 days after the last injection. The protocol used was as described by Walker et al., 2013. Dissected DG were enzymatically digested using The Neural Tissue Dissection Kit (Miltenyi), and following the manufacturer's instructions. After the last wash, the unstained pellet was resuspended in 1ml of DMEM:F12 without phenol red and filtered through a 40µm sieve. The YFP^{+} cells were analyzed using a FACS Aria Cell Sorter (BD Biosciences). DGs of animals not carrying the *Glast-CreERT2* allele were used for YFP gating and dead cells were excluded by propidium iodide staining (1ug/ml). Three to five animals of the same genotype were pooled for each sorting and considered as one n. 3000-cell samples were collected in DMEM:F12 + 1% BSA, pelleted down by centrifugation (2,800 rpm, 5 minutes) and 100µl of lysis buffer from the PicoPure RNA Isolation Kit (Life Technologies) was added for further processing (see below).

2.6 Laser capture microdissection

For laser capture microdissection (LCM) tissue collection, brains were removed after sacrifice, rapidly frozen in OCT on dry ice and stored at -80°C until further use. 14µm coronal sections spanning the length of the DG were cut in a cryostat (CM3050S, Leica) and placed on MembraneSlides (Zeiss). The SGZ of *WT*, *Ascl1^{neoflox}* and *Ascl1^{neo}cKO* animals was excised by a PLAM laser-capture

microdissection system (Zeiss) and manually collected in an adhesive cap (AdhesiveCap 200, ZEISS). 100µl of PicoPure RNA Isolation Kit lysis buffer (Life Technologies) was then added for further processing (see below).

2.7 Gene expression assays

2.7.1 RNA isolation

For studying gene expression after FACS or LCM sample collection, RNA was isolated after sample lysis was completed by incubating in the buffer mentioned above for 30 minutes at 42°C. If the procedure was not carried out immediately after, samples were kept at -80°C. In the case of FACS-sorted cells, the RNeasy Kit (Qiagen) was used to extract the RNA, and this was done as advised by the manufacturer. Each 3000-cell sample was eluted with 22µl of water. For LCM isolated samples, RNA was, instead, extracted using the PicoPure RNA Isolation Kit (Life Technologies), but also eluted with 22µl of water. Samples were immediately used for cDNA production or otherwise kept at -80°C until used.

2.7.2 cDNA production

RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RNA from each 3000-cell sample was pooled, and 20µl reactions were prepared with 9µl of RNA in each. The thermal programme used consisted of 60 minutes at 37°C and 5 minutes at 95°C. The cDNA from each independent sample was also pooled after generation.

2.7.3 Quantitative-PCR

Gene expression was detected using TaqMan Gene expression assays (Applied Biosystems) as described by the manufacturer and performed on a 7500 real time PCR system (Applied Biosystems). Data were analysed using standard protocols to calculate relative expression with the dCT method with *Gapdh* serving as an endogenous control. Each probe was performed in duplicates for at least 3 independent samples per group.

2.8 Statistical and bioinformatics analyses

Statistical analysis was conducted using a two-sample t test with equal variance using Prism software. A p-value of <0.05 was considered significant. All values represent mean values, and error bars represent the standard error of the mean (SEM).

GO analysis was conducted with Database for Annotation, Visualization, and Integrated Discovery (DAVID) using functional annotation clustering. Representative terms from the top-ranking clusters of GO terms are reported, all with p-value < 0.05 and false discovery rate (FDR) < 5%. See Andersen et al., 2014 for a description of ChIP-seq data generation and processing.

Chapter 3 Results

Ascl1 deletion in stem cells of the adult dentate gyrus

The proneural transcription factor Ascl1 is an essential regulator of neurogenesis in the embryonic brain, playing important roles in progenitor proliferation, specification and differentiation (Casarosa et al., 1999, Castro et al., 2011). Recent work has also established Ascl1 to be expressed in precursor cells of the neurogenic regions of the adult brain: the hippocampus and the SVZ. Importantly, lineage tracing experiments have shown Ascl1 to be expressed in a subset of self-renewing cells in these areas (Kim et al., 2011). Having in mind the aim of this work, which is that of studying the role of *Ascl1* in the DG of the hippocampus in adult mice, I will start this chapter by describing the expression of Ascl1 in this region. Next, I will move on to describe the conditional deletion of this factor in the DG, which is the main strategy used to understand its function in the adult brain. I will introduce the different mouse lines that I used to investigate the loss of *Ascl1* and briefly explain their advantages and disadvantages. I will then describe the main phenotype that arises from deleting *Ascl1* in self-renewing cells, and I will do this for the different mouse lines presented. And finally, in this chapter I will investigate the cell autonomy of the phenotype observed, since Ascl1 is expressed in more than one cell type.

3.1 Ascl1 is expressed in intermediate progenitors

Previous reports on the expression of Ascl1 in the adult DG have focused on its expression in IPCs, which comprise the majority of the cycling population in the SGZ, and are characterised by their SGZ localisation, their proliferative state and their lack of GFAP expression. Labelling of two month-old brains with a monoclonal antibody against Ascl1 together with the proliferation and activation markers Ki67 and MCM2 revealed that, indeed, the expression of this transcription factor, mostly confined to the SGZ, corresponds to proliferating cells (Figures 3-1A and 3-1B). Of all cells positive for Ascl1 94.32% ± 2.96% are also positive for MCM2. The same is not true for $MCM2^+$ cells, with only a small proportion of them being $Ascl1^+$ (15.65% ± 4.36).

Tbr2 is a well-characterised marker for late IPCs. Immunolabeling for Tbr2 and Ascl1 showed that $Tbr2^+$ cells are indeed localised to the SGZ and lack GFAP expression (Figure 3-1C). Among $Tbr2^+$ cells, there are two populations: one that co-expresses Ascl1 and another one that is negative for Ascl1 (Figure 3-1C, yellow

arrowhead and white arrow, respectively). Quantification of the Ascl1 population revealed that only a small fraction of them was positive for Tbr2 as well (8.91% ± 3.25%, Figure 3-1D), and none of them express markers of more mature cells in the lineage, including DCX and NeuN (Figure 3-1E and not shown). Therefore, it is likely that an early population of IPCs express Ascl1, together with Tbr2, and that as $Tbr2^+$ IPCs initiate the process of differentiation they lose expression of Ascl1.

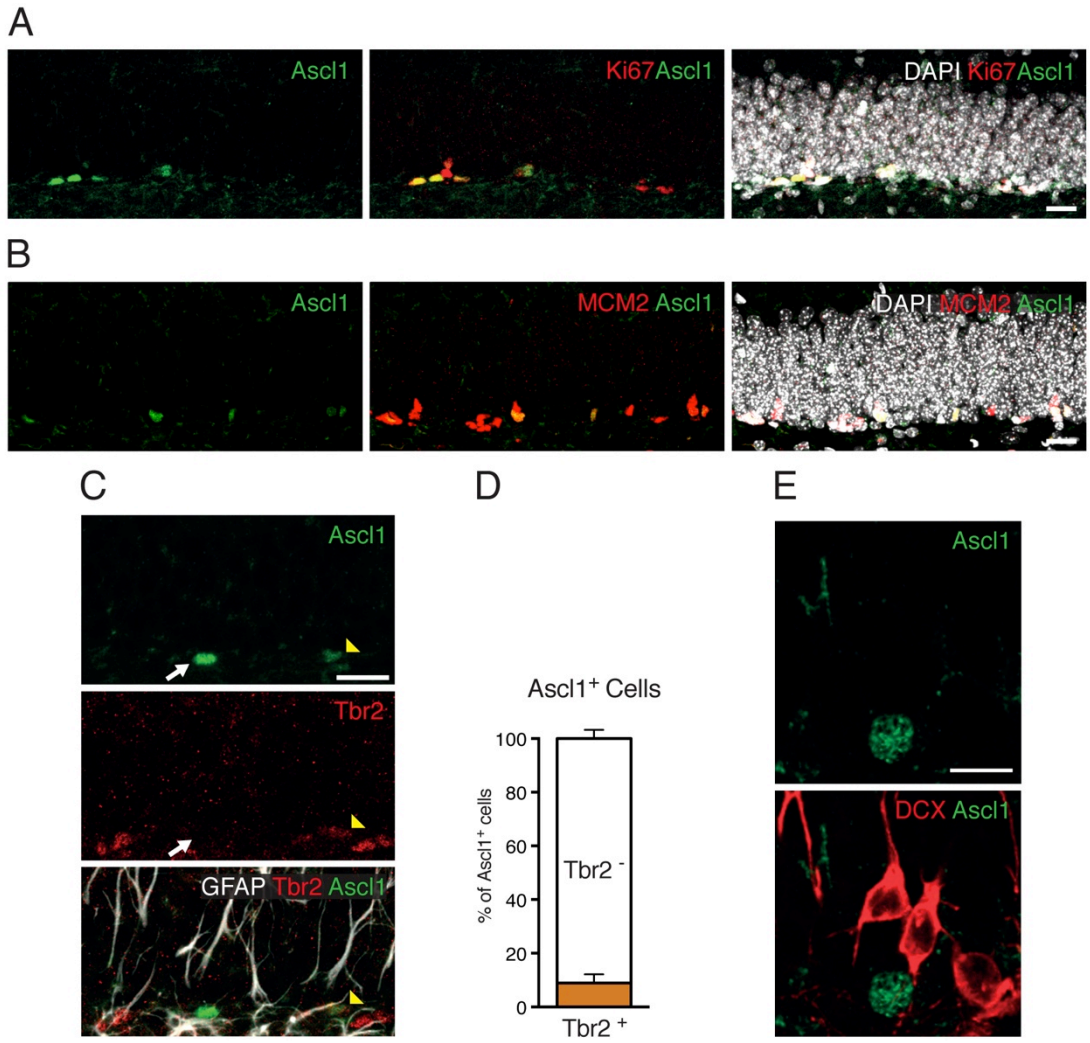

Figure 3-1 Ascl1 expression in intermediate progenitors in the SGZ of adult mice

(A and B) Labelling of Ascl1 shows that Ascl1 is expressed in the SGZ of the DG and that here it is colocalised with the proliferation marker Ki67 (A) as well as the activation marker MCM2 (B). Note the broader expression of MCM2 compared to Ki67 highlighting the earlier onset of expression of this marker. IPCs are the major cycling population in the DG, suggesting that Ascl1 is expressed in this population of cells. **(C and D)** Labelling for Ascl1 and the IPC marker Tbr2 and quantification of the percentage of Ascl1 cells that are $Tbr2^+$ confirms that Ascl1 is expressed by a subpopulation of IPCs. Only a small proportion of $Ascl1^+$ cells also expressed Tbr2 (yellow arrowhead in C), while a big majority of them are $Tbr2^-$. Most $Tbr2^-$ $Ascl1^+$ cells are also $GFAP^-$, indicating they are not stem cells (white arrow in C). (E) Staining for Ascl1 and DCX shows that Ascl1 is not expressed by immature neurons. n = 3 (C). Scale bars = 20µm (A, B, C and E).

3.2 Ascl1 is expressed in a subset of stem cells

Double labelling for Ascl1 and the stem cell marker GFAP revealed that a proportion of the total Ascl1-expressing cells have characteristics of RGLs (13.60% ± 0.42%). RGLs are characterised by the presence of a $GFAP^+$ radial process extending along the GL, and by the position of their cell bodies in the SGZ (see Figure 3-2A for an example of an RGL). Another important feature of stem cells in the hippocampus is that they are found mostly in a quiescent state. A small proportion of them, nevertheless, can be found to be active at any one time. Because Ascl1 is expressed, in its majority, in proliferating cells, I set to examine the population of $Ascl1^+$ RGLs in relation to those expressing either MCM2, Ki67 or BrdU after a 2-hour pulse. Only cells in S phase will have incorporated BrdU, while cells expressing Ki67 will correspond to a bigger population of cells from a late G_1 stage, and MCM2-expressing ones to an even bigger population, since this marker is expressed from an earlier G_1. Figure 3-2B shows the quantification for this experiment, and illustrates the increasing proportion of RGL cells that are positive for these three markers. As for Ascl1, I find it to be expressed in about 2% of the total RGL population, with a minimal proportion of them not expressing a proliferation marker. Together, these results show that there are 3 main populations of $Ascl1^+$ cells: a subpopulation of RGLs, which are mainly in an active state, a population of early IPCs that is negative for the later IPC marker Tbr2 and a small population of IPCs that also co-expresses Tbr2.

3.3 *Ascl1* deletion in stem cells of the adult hippocampus

3.3.1 Mouse lines used for deleting *Ascl1*

Mice carrying homozygous null alleles for *Ascl1* die at birth due to breathing defects, making the analysis of Ascl1 function at adult stages not feasible (Guillemot et al., 1993). To circumvent the lethality of these mice, a previous member of the laboratory generated transgenic mice carrying conditional mutant alleles for *Ascl1* (Pacary et al., 2011). We called these animals $Ascl1^{neoflox}$, for carrying a *PGK promoter-neo* cassette adjacent to the *Ascl1* locus (Figures 3-3C and 3-3D).

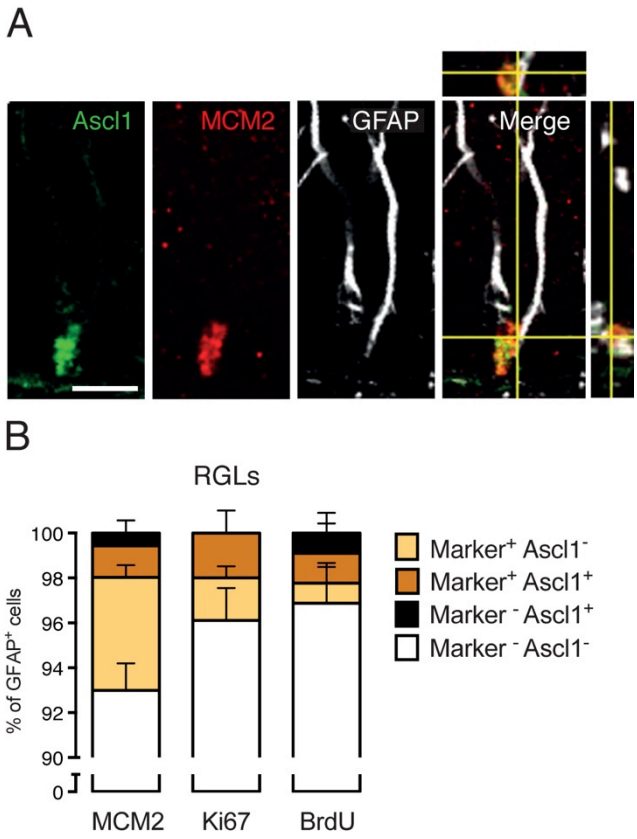

Figure 3-2 Ascl1 expression in hippocampal stem cells in the SGZ of adult mice

(A) Immonostaining for Ascl1 and the stem cell marker GFAP reveals that Ascl1 is expressed by radial GFAP-expressing RGLs in the DG. Colocalisation with MCM2 revealed that Ascl1$^+$ RGLs are in an active state. **(B)** Quantification showing the percentage of radial GFAP$^+$ cells expressing Ascl1 and the cell cycle markers MCM2 and Ki67 or incorporation of BrdU after a 2-hour pulse (marker). Ascl1 is expressed in approximately 2% of the total RGL population (dark orange and black bars), and most of these cells also express a cell cycle marker or were in the S phase at the time of BrdU administration (dark orange bars). Note the difference in the percentage of marker$^+$ populations. MCM2 is expressed from early G_1, while Ki67 is expressed later in G_1. BrdU will only mark cells in S phase at the time of BrdU injection. n = 3 for MCM2, Ki67 and BrdU. Scale bar = 10µm (A).

In order to delete *Ascl1* specifically in stem cells we made use of *Glast-CreERT2* mice, where TAM-inducible cre recombinase will be driven by the *Glast* promoter in RGLs and a proportion of IPCs (Mori et al., 2006). We also used mice carrying the *Rosa26-floxed stop-YFP* reporter transgene to follow the fate of recombined cells (Srinivas et al., 2001).

Tamoxifen administration for five consecutive days to two month-old mice and immunohistochemical analysis on the last day of injection revealed that a majority of GFAP$^+$ Nestin$^+$ radial cells (91.43% ± 3.48%) in a *WT* background induce the expression of YFP, indicating that cre has been activated and they have

therefore undergone recombination (Figures 3-3A and 3-3B). In animals carrying the *Ascl1*^{neoflox} alleles this recombination results in the deletion of *Ascl1* (*Ascl1*^{neo}*cKO*).

Due to concerns regarding the *Ascl1*^{neoflox} animals (see next section), I generated a new line where the neo cassette, flanked by *FRT* sites, was removed by FLP-induced recombination (Figure 3-3D). We called these animals *Ascl1*^{flox}; and as it was the case for the *Ascl1*^{neoflox} mice, administration of TAM to these animals leads to the loss of *Ascl1* from the genome (*Ascl1cKO*; see Figures 3-3C and 3-3D for exact genotypes and treatment of each line and the relationship between them).

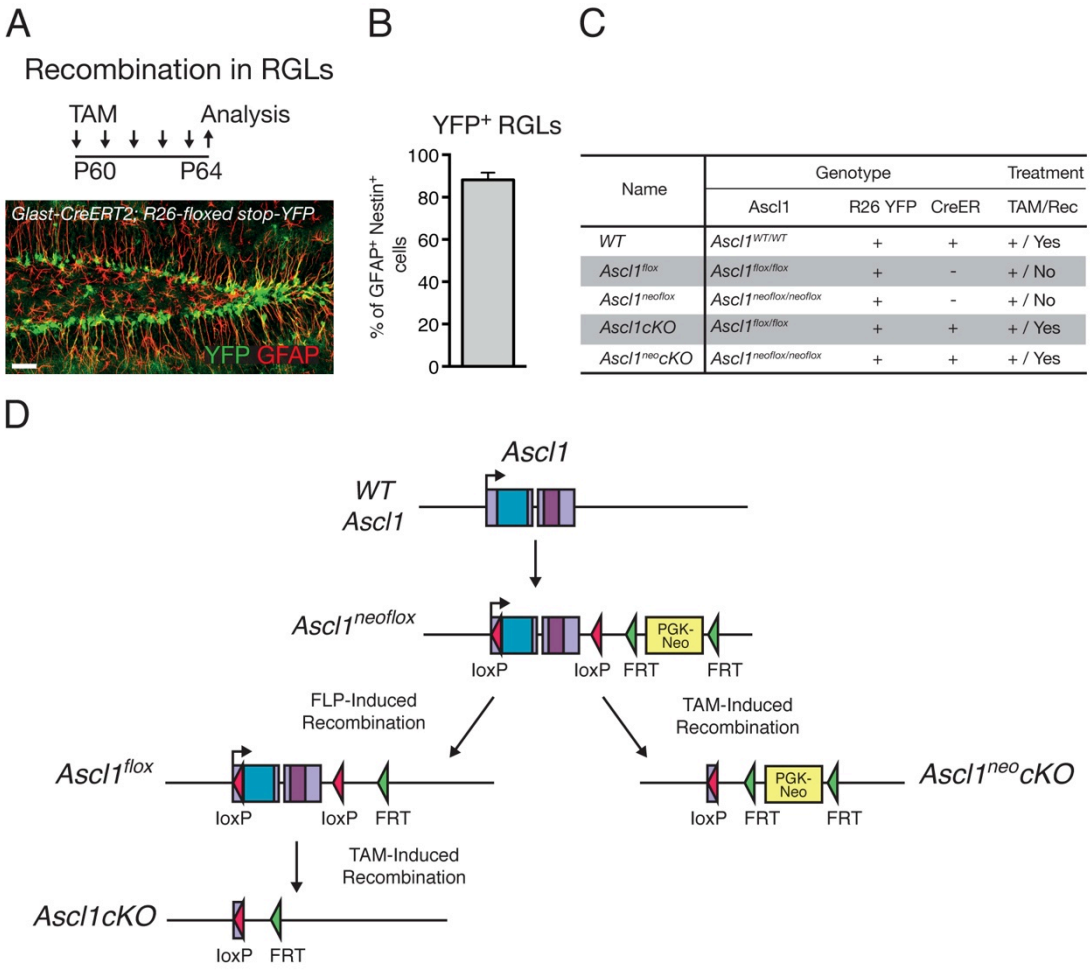

Figure 3-3 Depiction of mouse lines used for *Ascl1* deletion in the study (legend next page)

Figure 3-3 Depiction of mouse lines used for *Ascl1* deletion in the study

(A) Labelling for YFP and GFAP to illustrate the recombination efficiency in RGLs after administration of TAM for five consecutive days in in P60 *Glast-CreERT2* animals crossed with animals carrying the *Rosa26-floxed stop-YFP* reporter transgene. **(B)** Quantification of the recombination efficiency in these animals shows that a majority of RGLs, considered as $GFAP^+$ $Nestin^+$ radial cells, have undergone recombination and thus are YFP^+ after TAM injections at P60-P64. **(C)** Presentation of the main mouse lines used to study the effect of *Ascl1* deletion in the adult hippocampus showing their names (first column), the *Ascl1* allele they carry (*WT*, *flox* or *neoflox*, second column), the *Rosa26-floxed stop-YFP* (R26) reporter transgene they all carry (+, third column), whether they carry (+) or not carry (-) the *Glast-CreERT2* deleter allele (CreER, fourth column), and whether the TAM treatment they all received (TAM, +) results in recombination events leading to *Ascl1* deletion and expression of YFP (Rec, Yes or no, fifth column). **(D)** Scheme showing how the different mutant alleles for *Ascl1* were generated. The $Ascl1^{neoflox}$ was created by inserting a first *loxP* site at the start codon of *Ascl1* and a second one after the polyA signal at the 3' end of the *Ascl1* transcript. A *PGK promoter-neo* cassette flanked by *FRT* sites was also inserted at the 3' end (Pacary et al., 2011). TAM-induced cre activation results in recombination at the $Ascl1^{neoflox}$ allele to generate $Ascl1^{neo}cKO$ mice. FLP-induced recombination of $Ascl1^{neoflox}$ mice was used to generate $Ascl1^{flox}$ mice, which after TAM-induced recombination and deletion of the *PGK promoter-neo* cassette generate the *Ascl1cKO* mice. n = 3 (B). Scale bar = 40µm (A).

3.3.2 $Ascl1^{neoflox}$ animals show a hypomorphic phenotype

Expression analysis one month after TAM administration showed that, in *WT* animals, both recombined RGLs and their progeny express Ascl1. This expression is absent in $Ascl1^{neo}cKO$ mice (Figure 3-4A). Moreover, this analysis showed that animals that carry the $Ascl1^{neoflox}$ alleles, but do not have the cre recombinase in their genome, and are therefore unable to induce recombination, have lower levels of Ascl1 protein (Figure 3-4A, central panel).

Because the finding of lower levels of Ascl1 protein in animals carrying the $Ascl1^{neoflox}$ allele was unexpected, we went on to investigate the phenomenon further, and examined whether it might be due to reduced transcription of *Ascl1* at the locus. For this we made use of the laser capture microdissection (LCM) technique to dissect the SGZ of *WT*, $Ascl1^{neoflox}$ and $Ascl1^{neo}cKO$ animals (Figure 3-4B). We were then able to extract mRNA from these fragments and measure expression of *Ascl1* transcripts by RT-qPCR. This analysis confirmed that $Ascl1^{neoflox}$ animals have significantly reduced levels of *Ascl1* expression at the transcript level compared with *WT* mice. *Ascl1* expression is undetectable in $Ascl1^{neo}cKO$ mice, as expected (Figure 3-4C). These results suggest that the $Ascl1^{neoflox}$ is a hypomorphic allele, showing a reduction of gene activity (Nagy et al., 1998). These results bring about concerns regarding developmental defects in animals carrying this allele. We know that Ascl1 is crucial for embryonic development; hence the necessity to generate a conditional

line where expression of cre recombinase can be driven in an inducible manner and *Ascl1* can be deleted specifically at adult stages. *Ascl1*^{neo}*cKO* mice undergo development with defective *Ascl1* expression (i.e. they carry two *Ascl1*^{neoflox} alleles), and any phenotype that we see during adulthood might be a consequence of abnormal development. For this reason, the rest of the analysis will include *Ascl1*^{neoflox} mice as an additional experimental condition.

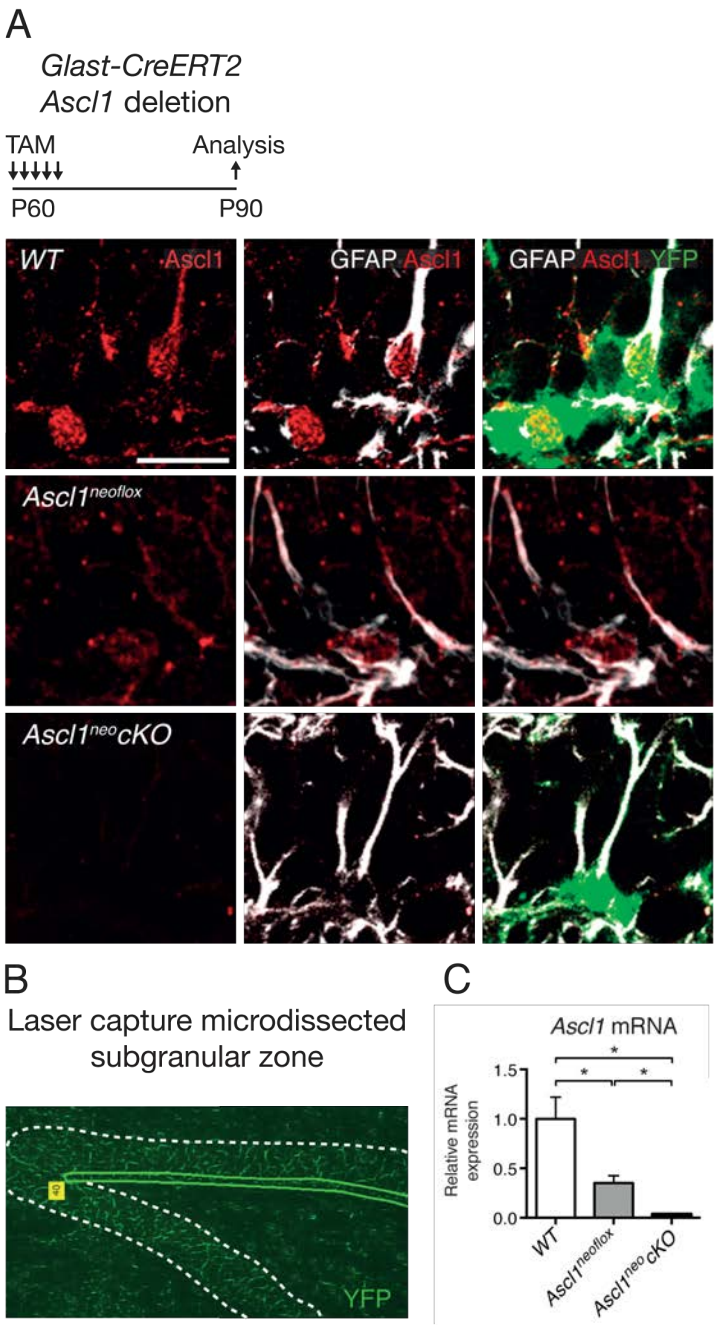

Figure 3-4 *Ascl1*^{neoflox} mice show reduced levels of *Ascl1* expression and Ascl1 protein (legend next page)

Figure 3-4 *Ascl1*^{neoflox} mice show reduced levels of *Ascl1* expression and Ascl1 protein

(A) Immunostaining showing Ascl1 expression one month after TAM administration in *WT*, *Ascl1*^{neoflox} and *Ascl1*^{neo}*cKO* mice. *WT* animals show expression of Ascl1 in YFP^{+} IPCs and RGLs, and this is absent in *Ascl1*^{neo}*cKO* mice. Ascl1 expression in *Ascl1*^{neoflox} mice is very weak or undetectable. **(B)** Image showing a laser capture microdissected SGZ (green rectangle) in the hippocampal DG (dashed line) of P90 animals that received TAM and P60. **(C)** Analysis of *Ascl1* transcripts by qPCR of laser capture microsidessected SGZ tissue shows that *Ascl1* expression is strongly and significantly reduced in *Ascl1*^{neoflox} mice compared to *WT* mice, and it is eliminated in *Ascl1*^{neo}*cKO* mice. Expression levels are normalized to *Gapdh* and are relative to *Ascl1* expression in *WT*. n = 3 for *WT*, *Ascl1*^{neoflox} and *Ascl1*^{neo}*cKO*. (C and D) Performed by Ayako Ito. * $p<0.05$. Scale bar = 20μm (A).

3.3.3 *Ascl1cKO* animals fail to completely delete *Ascl1*

We hypothesised that it was the *PGK promoter-neo* cassette that remained inserted on the 3' side of the *Ascl1* locus that was having an effect on the expression of this gene, since it has already been shown to be the case for others (Nagy et al., 1998, Meyers et al., 1998). For this reason I went on to cross *Ascl1*^{neoflox} and *actβ-Flp* animals to induce the excision of the *FRT*-flanked cassette (see Figure 3-3D). Ascl1 protein expression in the resulting *Ascl1*^{flox} animals was back to levels comparable to *WT* animals, demonstrating that it was, indeed, the flanking *PGK promoter-neo* sequence that was disrupting normal *Ascl1* expression (Figure 3-5, central panel).

Further analysis of these animals revealed, however, that Ascl1 expression is still present in *Ascl1cKO* animals in a proportion of cells that express YFP and have therefore recombined (Figure 3-5, bottom panel). This shows that there is an uncoupling between *Ascl1* deletion and YFP expression. Because the *Ascl1* conditional mutant allele and the *Rosa26-floxed stop-YFP* reporter transgene are in different chromosomes in the genome, cre-induced recombination of *Ascl1* and *YFP* are independent events. Moreover, for *Ascl1* deletion to be complete both alleles need to be deleted (see Figure 3-8, showing that heterozygous *Ascl1* mutant animals are comparable to *WT*), while having only one *Rosa26-floxed stop-YFP* allele undergoing recombination is enough for YFP to be expressed. Therefore, if the cre recombinase is not 100% efficient, it is possible for YFP to be expressed without *Ascl1* being deleted. Interestingly, we did not detect any uncoupling in the *Ascl1*^{neo}*cKO* mice (and the striking phenotype observed in these animals suggest that it is not a common event, see results below). This might be due to the fact that *Ascl1* expression in *Ascl1*^{neoflox} animals is already significantly lower compared to *WT*

mice, and recombination of only one of the alleles might be enough to abolish *Ascl1* function.

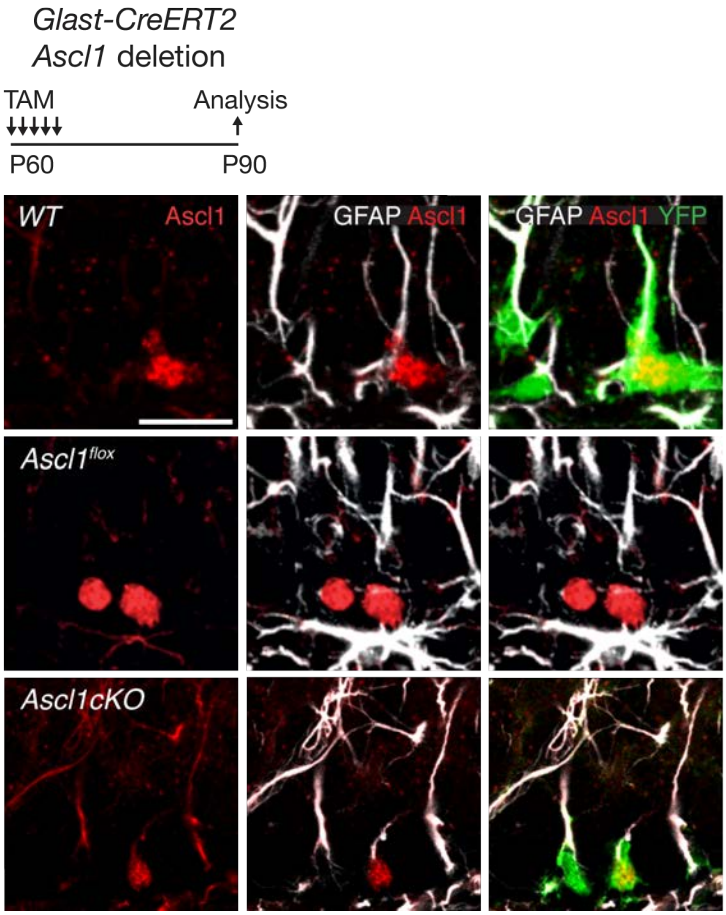

Figure 3-5 Incomplete *Ascl1* deletion in *Ascl1cKO* mice

Labelling for Ascl1, the stem cell marker GFAP and the recombination reporter YFP in P90 animals that received TAM at P60. *Ascl1*^{flox} mice, which do no longer have the *PGK promoter-neo* cassette, show comparable levels of Ascl1 expression to *WT* mice. In *Ascl1cKO* animals, Ascl1^{+} cells that are also YFP^{+} can be found indicating that recombination of the *Ascl1*^{flox} allele is not complete.

3.4 *Ascl1* deletion results in a loss of proliferation in *Ascl1*^{neo}*cKO* mice

3.4.1 Effect on progenitor proliferation

Since, as described in the previous chapter, I found a majority of Ascl1-expressing cells to be in a proliferative state, the first thing to do after removing *Ascl1* from the genome was to check for the presence of proliferation. Strikingly, TAM i.p. injection for 5 days at P60 and analysis at P90 showed that deletion of *Ascl1* in *Ascl1*^{neo}*cKO* mice results in a complete loss of dividing cells in the DG (Figure 3-6A),

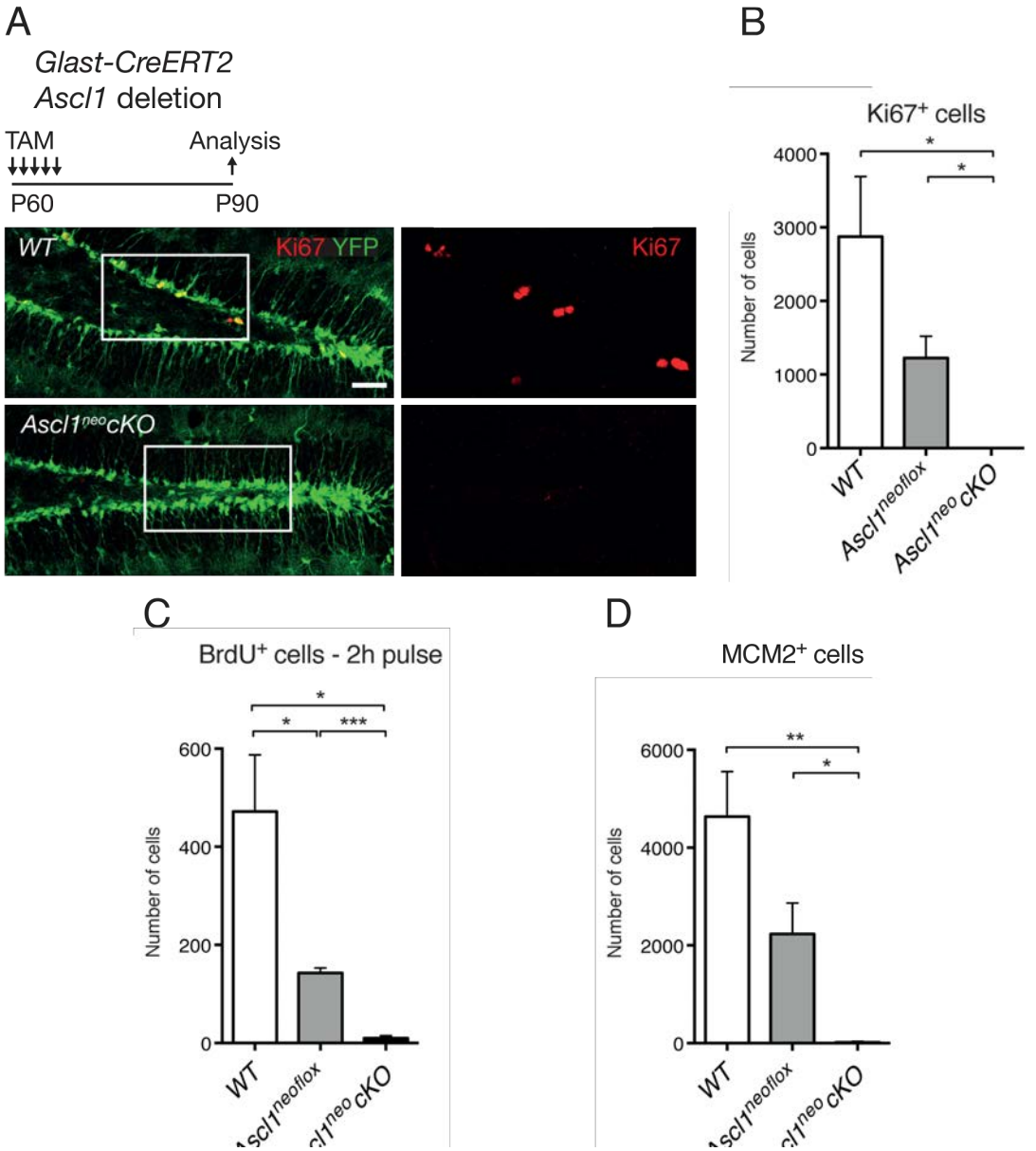

Figure 3-6 Block of proliferation and activation by conditional deletion of *Ascl1*

(A and B) Animals received TAM from P60 for five consecutive days and analysis was performed at P90 to examine proliferation after *Ascl1* deletion. Labelling for the proliferation marker Ki67 and quantification of the total number of Ki67$^+$ cells in the DG revealed that proliferation here is blocked. *Ascl1^{neo}cKO* animals show a complete absence of Ki67$^+$ cells, while *Ascl1^{neoflox}* animals, where *Ascl1* expression is reduced, show, accordingly, a reduction in the number of proliferating cells compared to *WT* mice. **(C and D)** The same result was observed after a 2-hour pulse of BrdU, and with immunolabeling for the activation marker MCM2. n = 3 (C), 4 (B) and 5 (D) for *WT*; 3 (C) and 4 (B, D) for *Ascl1^{neoflox}*; 3 (B, C) and 4 (D) for *Ascl1^{neo}cKO*. * p<0.05, ** p<0.01, *** p<0.001. Scale bar = 40µm (A).

as shown by the absence of Ki67$^+$ and BrdU-incorporating cells after a 2 hour pulse (Figures 3-6B and 3-6C). Moreover, by quantifying the number of proliferating cells in *Ascl1^{neoflox}* animals I found that reduced *Ascl1* levels is also translated into a

reduced number of dividing cells compared to *WT* animals (*WT* versus *Ascl1*^{neoflox} versus *Ascl1*^{neo}*cKO*, 2875 ± 815 versus 1225 ± 297 versus 0 Ki67^{+} cells; 471 ± 115 versus 142 ± 10.2 versus 9.78 ± 4.90 BrdU^{+} cells; Figures 3-6B and 3-6C).

Next, to discard the possibility that cells were halted at an early stage of the cell cycle that is not marked by Ki67, I performed immunostaining against MCM2, which marks cells from an early stage of the G_1 phase of the cycle (Niu et al., 2011, Stoeber et al., 2001)). This staining showed that there are no cells entering the cell cycle, since I find again a complete loss of MCM2^{+} cells. Similarly, I find less cells expressing MCM2 in *Ascl1*^{neoflox} animals (*WT* versus *Ascl1*^{neoflox} versus *Ascl1*^{neo}*cKO*, 4635 ± 921 versus 2233 ± 632 versus 19.65 ± 19.65 MCM2^{+} cells; Figure 3-6D).

3.4.2 Effect on RGL proliferation

Having observed a loss of the dividing population in the adult DG after deleting *Ascl1*, I then went on to look specifically at the proliferative state of RGLs. The majority of the cycling cells at one time will correspond to IPCs and only between 5% and 10% of these represent the stem cells. For analysis purposes, I defined an RGL as a YFP^{+} cell that has a GFAP^{+} radial process extending along the GL and whose cell body is positioned on the SGZ (Figure 3-7A). *Ascl1* deletion following the same protocol as described in the previous section, revealed a total block in RGL proliferation. Both Ki67 and the earlier marker MCM2 were absent from RGLs (*WT* versus *Ascl1*^{neoflox} versus *Ascl1*^{neo}*cKO*, 161 ± 48.5 versus 66.0 ± 29.6 versus 0 Ki67^{+} RGLs; 281 ± 70.1 versus 126 ± 26.3 versus 0 MCM2^{+} RGLs; Figures 3-7B and 3-7C). This suggests that the loss of the cycling population in the DG stems from an inability of RGLs to enter the cell cycle and proliferate.

Finally, to confirm that the absence of Ki67^{+} and MCM2^{+} cells is not due to a failure in detection due to cells dividing slower or more infrequently, I carried out a long-term BrdU retention paradigm. This paradigm is designed to label rarely dividing RGLs that are capable of retaining BrdU, administered for 10 consecutive days, after a 20 day chase period (Figure 3-7D). BrdU-label retaining cells were present in *WT* and, albeit in reduced numbers, in *Ascl1*^{neoflox} mice, but they were again completely absent in *Ascl1*^{neo}*cKO* mice (*WT* versus *Ascl1*^{neoflox} versus

Ascl1^{neo}*cKO*, 43.8 ± 6.2 versus 9.7 ± 4.9 versus 0 label-retaining RGLs; Figure 3-7D). This result shows that *Ascl1*-deleted RGLs are unable to incorporate BrdU, and thus confirms that RGLs do not enter the cell cycle in the absence of *Ascl1*.

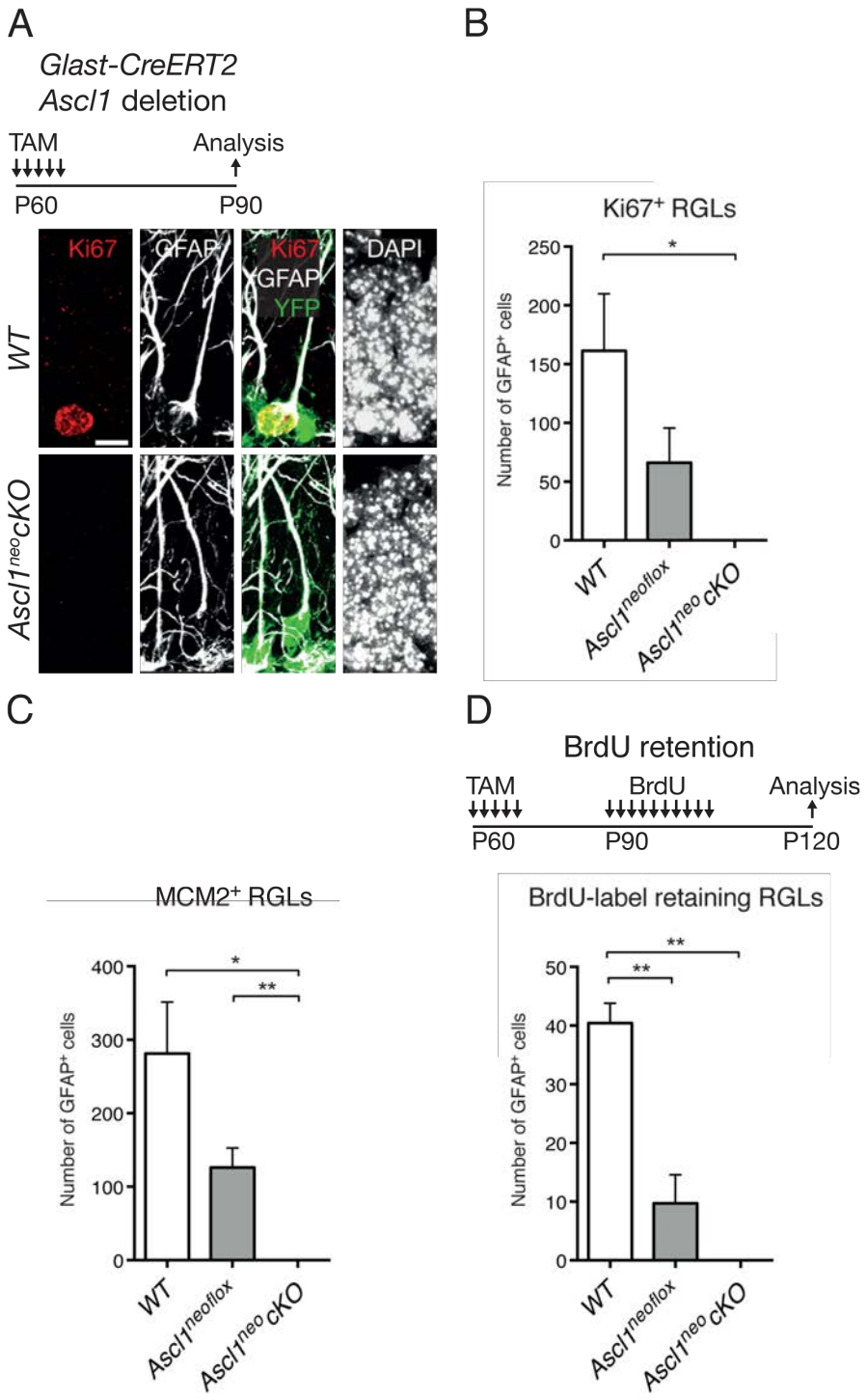

Figure 3-7 Block of hippocampal stem cell proliferation and activation by conditional deletion of *Ascl1* (legend next page)

Figure 3-7 Block of hippocampal stem cell proliferation and activation by conditional deletion of *Ascl1*

(A) Animals received TAM from P60 for five consecutive days and analysis was performed at P90 to examine stem cell proliferation after *Ascl1* deletion. Stem cells are considered those cells that possess a $GFAP^+$ radial process extending along the GL. Proliferating stem cells are considered those with a proliferation marker-positive nucleus. **(B)** Quantification of the total number of $Ki67^+$ $GFAP^+$ radial cells in the DG showed that stem cells that have inactivated *Ascl1* in *Ascl1*^{neo}*cKO* animals do not proliferate. Similarly, mice that have reduced levels of *Ascl1* expression show a reduction in the total number of $Ki67^+$ stem cells. **(C)** Immunostaining and quantification for the activation marker MCM2 also showed that stem cells in *Ascl1*^{neo}*cKO* mice fail to activate, and that in *Ascl1*^{neoflox} mice, compared to *WT* animals, there are significantly less active RGLs. **(D)** Administration of BrdU for 10 days followed by a three-week chase period shows there are no BrdU-label retaining RGLs in *Ascl1*^{neo}*cKO* mice confirming that stem cells in these animals do not divide. n = 4 (B) and 5 (C, D) for *WT*; 3 (D) and 4 (B, C) for *Ascl1*^{neoflox}; 3 (B, D) and 4 (C) for *Ascl1*^{neo}*cKO*. * $p<0.05$, ** $p<0.01$. Scale bar = 10µm (A).

3.4.3 Changes in *Ascl1* expression levels affect proliferation

The results shown so far suggest that Ascl1 is essential for RGLs to proliferate. They also suggest, however, that it is not just its presence that is important, but also the level of expression it is present at. *Ascl1*^{neoflox} animals show significantly lower levels of *Ascl1* mRNA and protein expression, and this is translated into reduced proliferation (see Figure 3-7). To further explore this idea, I crossed *Ascl1*^{neoflox} animals with animals carrying a null *Ascl1* allele (*Ascl1 Δ*; Guillemot et al., 1993). In this way I generated two more lines (*WT/Δ* and *Ascl1*$^{neoflox/Δ}$) that have intermediate levels of *Ascl1* expression between a *WT* and an *Ascl1*^{neoflox} and between the *Ascl1*^{neoflox} and the *Ascl1*^{neo}*cKO*, respectively (see Figure 3-8A for a table with genotypes). This approach could give me more evidence on the importance of *Ascl1* expression levels.

Analysis of the number of $Ki67^+$ cells in the DG of these animals one month after TAM administration showed that indeed, the level of *Ascl1* expression is correlated with the level of proliferation (Figure 3-8B). Reducing the level of *Ascl1* by the use of the different transgenic mice lines reduced the number of dividing cells. Interestingly, having at least one untouched *Ascl1* allele is enough for having normal proliferation, since I saw no significant difference between the number of $Ki67^+$ cells in *WT* and *WT/Δ* animals (*WT* versus *WT/Δ* versus *Ascl1*^{neoflox} versus *Ascl1*$^{neoflox/Δ}$ versus *Ascl1*^{neo}*cKO*, 2875 ± 815 versus 2032 ± 475 versus 1225 ± 297 versus 384 ± 147 versus 0 $Ki67^+$ cells; Figure 3-8B). This suggests that there is a threshold effect: a minimal level of *Ascl1* expression is required for proliferation to go on as normal,

A

Name	Genotype			Treatment
	Ascl1	*R26 YFP*	*CreER*	TAM / Rec
WT	$Ascl1^{WT/WT}$	+	+	+ / Yes
WT/Δ	$Ascl1^{WT/\Delta}$	+	+	+ / Yes
$Ascl1^{neoflox}$	$Ascl1^{neoflox/neoflox}$	+	-	+ / No
$Ascl1^{neoflox/\Delta}$	$Ascl1^{neoflox/\Delta}$	+	-	+ / No
$Ascl1^{neo}cKO$	$Ascl1^{neoflox/neoflox}$	+	+	+ / Yes

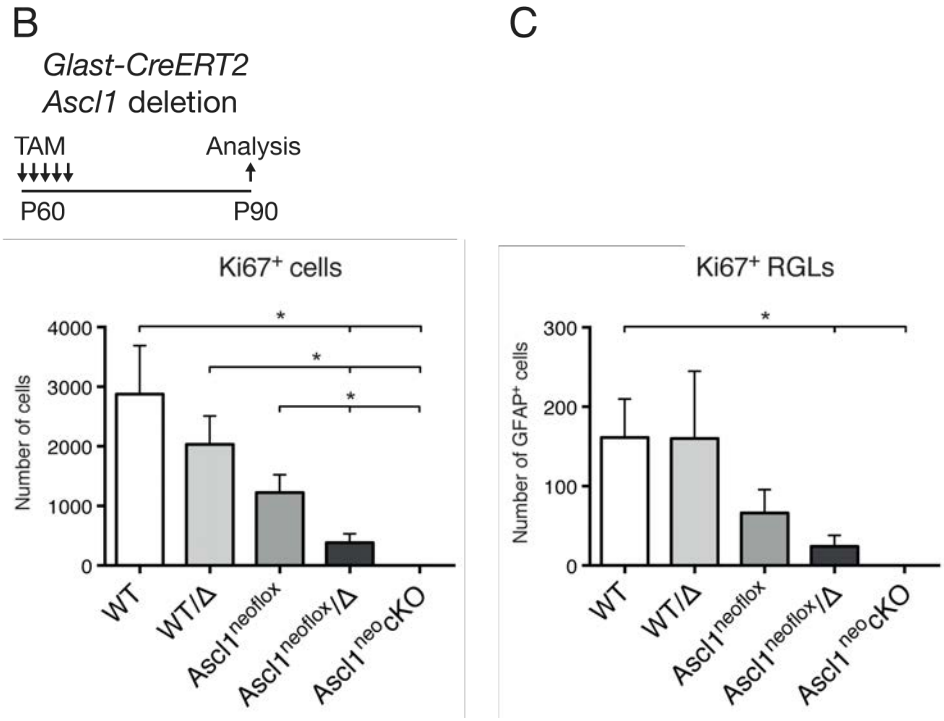

Figure 3-8 Correlation of *Ascl1* expression levels and proliferation in the DG

(A) Presentation of the mouse lines used to study the results of reducing Ascl1 levels on proliferation showing their names (first column), the *Ascl1* allele they carry (*WT*, *Δ,* or *neoflox*, second column), the *Rosa26-floxed stop-YFP* (R26) reporter transgene they all carry (+, third column), whether they carry (+) or not carry (-) the *Glast-CreERT2* deleter allele (CreER, fourth column), and whether the TAM treatment they all received (TAM, +) results in recombination events leading to *Ascl1* deletion and expression of YFP (Rec, Yes or no, fifth column). The lines are ordered from top to bottom in relation to their expected levels of *Ascl1* expression. **(B and C)** TAM administration at P60 and analysis one month later showed that *Ascl1* levels correlate with the total number of $Ki67^+$ cells and $Ki67^+$ RGLs in the DG. No difference is observed in the number of cells between two or one *WT* Ascl1 alleles (WT and WT/ *Δ*), indicating that one normal allele is enough for correct proliferation. However, a bigger reduction in *Ascl1* levels is translated into a bigger reduction in proliferation. n = 3 for *WT/ Δ* and 4 for *WT*, $Ascl1^{neoflox}$, $Ascl1^{neoflox/\Delta}$ and $Ascl1^{neo}cKO$. * $p<0.05$.

below this threshold of *Ascl1* expression, which we reach with our $Ascl1^{neoflox}$ animals, proliferation is disrupted, and this is amplified as expression continues to decrease. The same is true for the number of $Ki67^+$ RGLs in the DG of these different lines (*WT* versus *WT/Δ* versus $Ascl1^{neoflox}$ versus $Ascl1^{neoflox/\Delta}$ versus

Ascl1^{neo}*cKO*, 161 ± 48.5 versus 160 ± 84.7 versus 66.0 ± 29.6 versus 24.0 ± 13.9 versus 0 Ki67^{+} RGLs; Figure 3-8C).

3.5 *Ascl1* deletion results in a loss of proliferation in *Ascl1cKO* mice

3.5.1 Effect on progenitor proliferation

Next I went on to analyse the phenotype of the *Ascl1cKO* mice, which do not carry the *PGK promoter-neo* sequence and have therefore levels of *Ascl1* that are comparable to *WT* animals until they undergo cre-induced recombination. The phenotype in *Ascl1*^{neo}*cKO* animals appears to be very clear; however, because of concerns regarding the development of *Ascl1*^{neoflox} animals with reduced *Ascl1* levels, it is important to also study *Ascl1* deletion in animals that have normal *Ascl1* expression during development. For this I followed the same procedure than before, and I quantified the number of Ki67^{+} or MCM2^{+} cells present in the DG of *WT*, *Ascl1*^{flox} and *Ascl1cKO* mice. I found, in a similar manner, a very significant reduction in the number of cells that are dividing with both Ki67 and MCM2 in *Ascl1cKO* animals when compared to *WT* and *Ascl1*^{flox} mice (*WT* versus *Ascl1*^{flox} versus *Ascl1cKO*, 1713 ± 205 versus 2651 ± 129 versus 316 ± 87.9 Ki67^{+} cells; 2656 ± 42.3 versus 6048 ± 1130 versus 480 ± 33.9 MCM2^{+} cells; Figures 3-9A and 3-9B). The reduction in this case is not as striking as it was in the case of the *Ascl1*^{neo}*cKO* mice, and this is no surprise, considering that I had seen an uncoupling of *Ascl1* and *YFP* recombination in these animals (see Figure 3-5).

As expected, the number of proliferating cells in *Ascl1*^{flox} animals, which carry *neo*-less *Ascl1* floxed alleles but no cre recombinase, is not reduced, confirming that Ascl1 expression in them is back to normal. Surprisingly, however, I found the number of cells in these animals to be actually significantly increased when compared to *WT* mice (Figures 3-9A and 3-9B). The only difference between these two lines is the presence or absence of the cre recombinase, suggesting that having an active cre can affect proliferation.

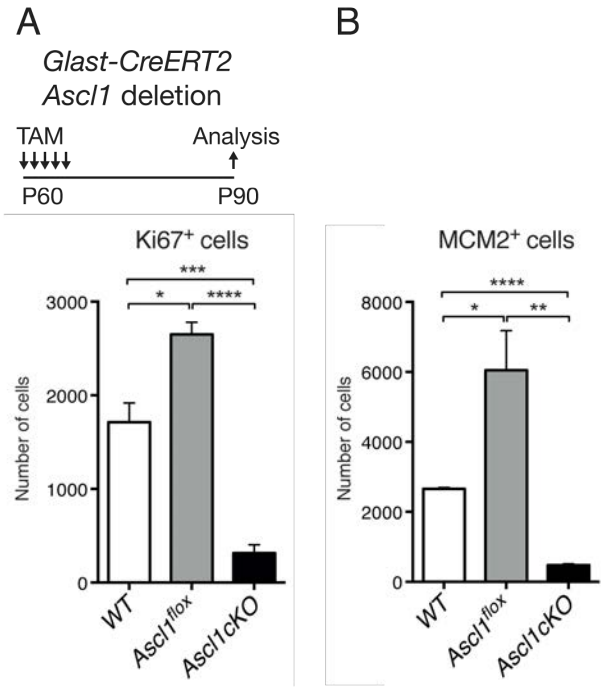

Figure 3-9 Block of proliferation and activation in *Ascl1cKO* mice

(A and B) Administration of TAM for five days and analysis one month later to animals that have undergone Flp-induced recombination and have therefore deleted the *PGK promoter-neo* cassette that was disrupting *Ascl1* expression shows that deletion of *Ascl1* in these animals also results in a block of proliferation and activation. The quantification of the total number of $Ki67^+$ (A) and $MCM2^+$ (B) per DG is shown. *Ascl1cKO* animals have significantly reduced numbers of both $Ki67^+$ and $MCM2^+$ cells compared to *WT* and $Ascl1^{flox}$, whose *Ascl1* levels are back to normal. $Ascl1^{flox}$ animals also show an increased number of dividing cells compared to *WT* mice. n = 3 for *WT* and $Ascl1^{flox}$; 5 for *Ascl1cKO*. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.5.2 Effect on RGL proliferation

When studying the presence of proliferation specifically in RGLs in *Ascl1cKO* animals I found, again, that RGLs that have deleted *Ascl1* fail to enter the cell cycle, and this is shown both by Ki67 and MCM2 expression (*WT* versus $Ascl1^{flox}$ versus *Ascl1cKO*, 125 ± 39.6 versus 165 ± 47.4 versus 9.6 ± 9.6 $Ki67^+$ RGLs; 112 ± 16.0 versus 224 ± 57.7 versus 28.8 ± 19.2 $MCM2^+$ RGLs; Figures 3-10A and 3-10B). As it was the case for total proliferation, the reduction in *Ascl1cKO* mice is not complete.

Due to this uncoupling in the recombination between the $Ascl1^{flox}$ and RYFP alleles, and because no developmental defects were observed (Andersen et al., 2014; and see Discussion, chapter 7), I will be using the $Ascl1^{neo}cKO$ animals for most of

the analysis. In experiments where the use of *Ascl1*^{neo}*cKO* animals risks biasing the results, the analysis will be performed with both lines.

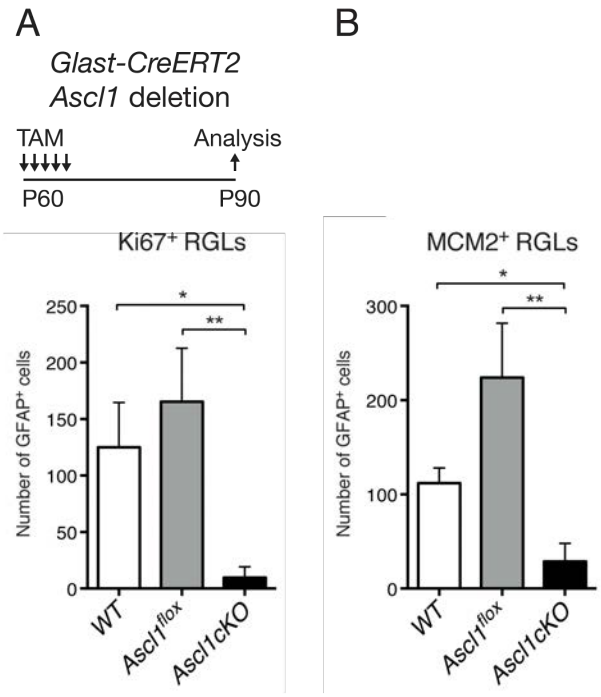

Figure 3-10 Block of proliferation and activation in hippocampal stem cells in *Ascl1cKO* mice

(A and B) Administration of TAM for five days to adult mice and analysis one month later showed that the total number of Ki67^{+} and MCM2^{+} RGLs is greatly reduced in *Ascl1cKO* mice. No significant difference is found in RGL numbers between *WT* and *Ascl1*^{flox} animals. n = 3 for *WT* and *Ascl1*^{flox}; 5 for *Ascl1cKO*. * $p<0.05$, ** $p<0.01$.

3.6 Ascl1 acts in a cell-autonomous manner

3.6.1 *Ascl1*-deleted cells do not proliferate from an early time-point

The data in this chapter strongly indicate that *Ascl1* is an essential factor required for the RGLs to enter the cell cycle and divide to generate more committed progeny. We know, nonetheless, that Ascl1 is expressed by IPCs as well as RGLs. There is a possibility, therefore, that the loss of RGL proliferation is a secondary consequence of an effect of *Ascl1* on IPCs. To investigate this possibility I first performed a short-term analysis, where RGL proliferation was quantified after the last TAM injection. In this way, by carrying out the analysis straight after TAM administration, non-recombined proliferating IPCs will still be present (Figure 3-11A), and if they were

exerting a positive effect on RGL proliferation, I would be able to see some dividing, rescued RGLs. Quantification of the percentage of RGLs that is $Ki67^+$ in P64 animals showed that there are no stem cells dividing even at this early time point in *Ascl1^{neo}cKO* mice (*WT* versus *Ascl1^{neo}cKO*, 1.8% ± 0.2% versus 0% $Ki67^+$ RGLs; Figures 3-11B and 3-11C). This suggests that the effect that we see in RGLs is cell autonomous and not a secondary consequence derived from the IPCs.

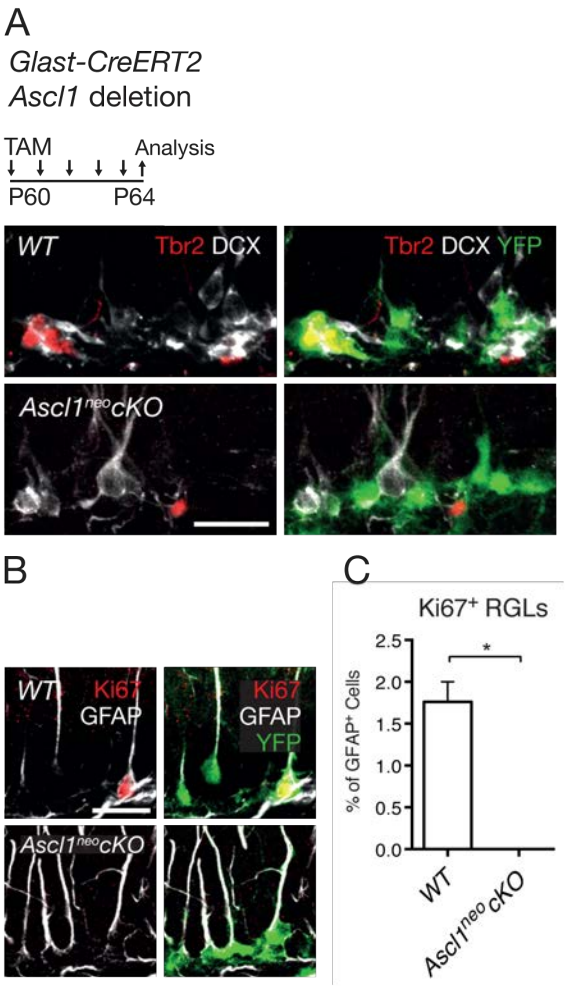

Figure 3-11 Short-term block of RGL proliferation in *Ascl1^{neo}cKO* mice

(A) Administration of TAM to P60 animals and analysis straight after the last of five injections results in an environment where recombined RGLs in *Ascl1^{neo}cKO* mice are surrounded by non-recombined IPCs and neuroblasts. This is shown by immunolabeling for the IPC marker Tbr2, the neuroblast marker DCX and the reporter YFP, which are present in both *WT* and *Ascl1^{neo}cKO* animals **(B and C)** Immunolabeling for GFAP, Ki67 and YFP and quantification of the percentage of Ki67-expressing cells in the $GFAP^+$ YFP^+ radial population show that despite the presence of *WT* progeny, RGLs in *Ascl1^{neo}cKO* do not proliferate shortly after *Ascl1* deletion. * $p<0.05$. Scale bars = 20µm (A, B).

3.6.2 *Ascl1*-deleted cells do not proliferate in the presence of progeny

To further validate the cell-autonomy of *Ascl1* on RGLs, I carried out a mosaic deletion of *Ascl1*, where I administered only one TAM injection as opposed to the five that I normally administer. In this way only a proportion of the stem cells will undergo recombination, while the rest would still be able to generate progeny (Figure 3-12A). Following the logic of the experiment above, if proliferating progeny were required for RGLs to divide, this set up would allow me to see any rescued RGLs. Quantification of $Ki67^+$ RGLs that are either YFP^- or YFP^+ in *WT* and $Ascl1^{neo}cKO$ mice showed that this is not the case. While in *WT* animals I was able to find both YFP^- and YFP^+ dividing RGLs, no YFP^+ RGL was positive for Ki67 in $Ascl1^{neo}cKO$ mice (YFP^- versus YFP^+, 81.5 ± 18.5 versus 161 ± 37.1 cells in *WT*; 47.6 ± 14.0 versus 0 cells in $Ascl1^{neo}cKO$; Figures 3-12B and 3-12C). This effect was independent of the recombination efficiency (Figure 3-12D).

I next performed the same mosaic analysis in *Ascl1cKO* mice to discard any possible experimental confound due to different backgrounds between the *WT* and $Ascl1^{neo}cKO$ mice. Quantification of the percentage of $Ki67^+$ or $MCM2^+$ RGLs that are either YFP^+ or YFP^- showed that *Ascl1*-deficient stem cells are unable to divide regardless of the context (YFP^- versus YFP^+, 0.4% ± 0.2% versus 0.07% ± 0.07% $Ki67^+$ RGLs; 0.7% ± 0.4% versus 0% $MCM2^+$ RGLs; Figures 3-12E and 3-12F). Here we also note the probable presence of uncoupled recombination, with a small proportion of YFP^+ RGLs being $Ki67^+$ (Figure 3-12E). Altogether these results indicate that *Ascl1* acts cell-autonomously to promote RGL proliferation in the DG of adult mice.

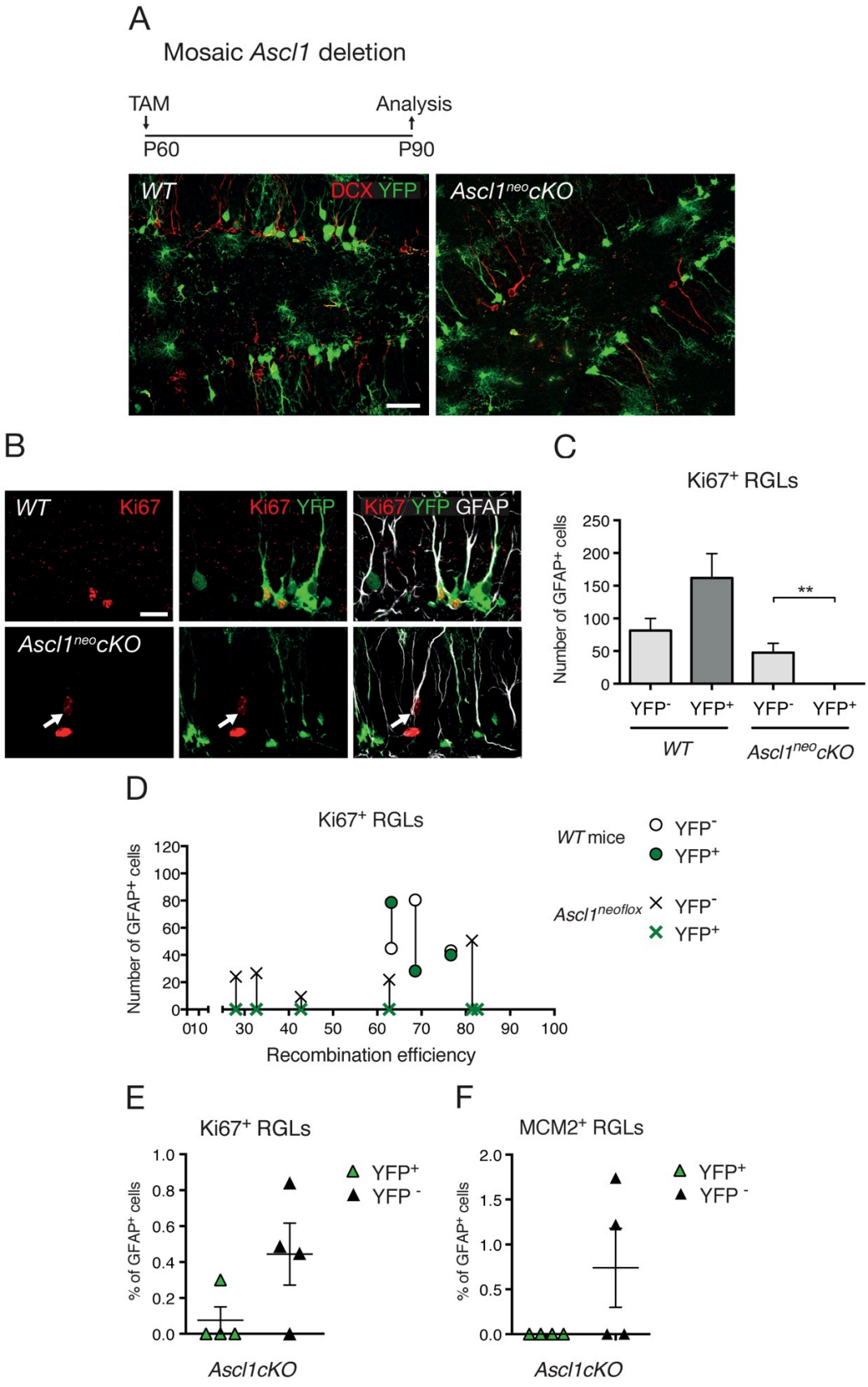

Figure 3-12 Cell autonomous function of *Ascl1* in RGLs after mosaic deletion (legend next page)

Figure 3-12 Cell autonomous function of *Ascl1* in RGLs after mosaic deletion

(A) Only one injection of TAM at P60 results in a mosaic *Ascl1* deletion with just a proportion of RGLs recombined. Immunolabeling for YFP and the neuroblast marker DCX illustrate this mosaic deletion and show that non-deleted precursors giving rise to progeny are still present in *Ascl1^{neo}cKO* mice. **(B)** Staining for the RGL marker GFAP, the proliferation marker Ki67 and YFP to visualise the active stem cell population show that while YFP$^-$ (non-recombined) RGLs can still proliferate in *Ascl1^{neo}cKO* mice (white arrow, bottom panel), RGLs that have deleted *Ascl1* (recombined, YFP$^+$) do not proliferate. **(C)** Quantification of the number of Ki67-expressing GFAP$^+$ radial cells in *WT* and *Ascl1^{neo}cKO* mice confirms that in a *WT* background both YFP$^+$ and YFP$^-$ RGLs exist, but in *Ascl1^{neo}cKO* animals, only YFP$^-$ Ki67$^+$ cells are found. **(D)** The loss of proliferation in *Ascl1^{neo}cKO* RGLs does not depend on the recombination efficiency. This is shown by plotting the total number of Ki67$^+$ RGLs (vertical axis) against the recombination efficiency as the ratio of YFP$^+$ RGLs to total RGLs (horizontal axis). Each *WT* and *Ascl1^{neo}cKO* mouse studied is plotted separately and the number of recombined (YFP$^+$) and non-recombined (YFP$^-$) RGLs for each mouse is linked by a vertical line. **(E and F)** Quantification of the percentage of Ki67 or MCM2-expressing GFAP$^+$ radial cells in *Ascl1cKO* mice in recombined (YFP$^+$) and non-recombined (YFP$^-$) RGLs. In animals that have undergone recombination to delete the *PGK promoter-neo* cassette the same result is observed; YFP$^+$ cells divide. Recombination efficiency in the RGL population for these experiments was between 12% and 40%. Note that expression of Ki67 in one YFP$^+$ RGL in (E) is likely due to the uncoupling of recombination between *Ascl1* and the *Rosa26 YFP* reporter alleles. n = 3 (C, D) for *WT*, 6 (C, D) for *Ascl1^{neo}cKO*; 4 (E, F) for *Ascl1cKO*. ** $p<0.01$. Scale bars = 20µm (B), 40µm (A).

Chapter 4 Results

Fate of *Ascl1*-deleted cells

Data so far has demonstrated that *Ascl1*-deficient RGLs are unable to divide and that this is a cell-autonomous effect. Next, I set out to determine the consequences of a loss of proliferation in RGLs in the DG. If stem cells do not proliferate then I hypothesise they do not generate any progeny, so first I analysed the neurogenic and astrogliogenic output of *Ascl1*-deleted cells. In this chapter I will also describe the fate of RGLs after *Ascl1* deletion: is *Ascl1* involved in maintaining the stem cell properties of RGLs, or does it exclusively affect their ability to proliferate? And finally, as an extension of these two sections, in this chapter I will go on to determine what is the long-term effect of *Ascl1* deletion.

4.1 *Ascl1* deletion results in a block of neurogenesis

One obvious question to ask having completely blocked RGL divisions by the deletion of *Ascl1* is whether any new neurons are still being generated. To answer this question I deleted *Ascl1* in two-month old mice by administering TAM for 5 days, and looked for the expression of markers characteristic of more committed cells in the neurogenic lineage one month later. This time frame is enough to allow cells generated at P60 to differentiate into neurons.

The lack of a proliferating population shown in the previous chapter suggests that there is indeed an absence of IPCs. To confirm this I performed immunolabeling for the IPC marker Tbr2, and I assuredly found no $Tbr2^+$ cells in *$Ascl1^{neo}$cKO mice*. Interestingly, I saw no difference in the number of $Tbr2^+$ cells between *WT* and *$Ascl1^{neoflox}$* mice (*WT* versus *$Ascl1^{neoflox}$* versus *$Ascl1^{neo}$cKO*, 1169 ± 202 versus 1089 ± 262 versus 30.0 ± 22.9 cells; Figures 4-1A and 4-1B). This is surprising considering that I found a significant reduction in the number of both $Ki67^+$ and $MCM2^+$ cells, as well as in the number of BrdU-incorporating cells in these mice (see Figure 3-6). This raises the question of whether this result is accurate or a technical artefact, which might be possible taking into account the knowledge of the variable nature of the Tbr2 immunostaining.

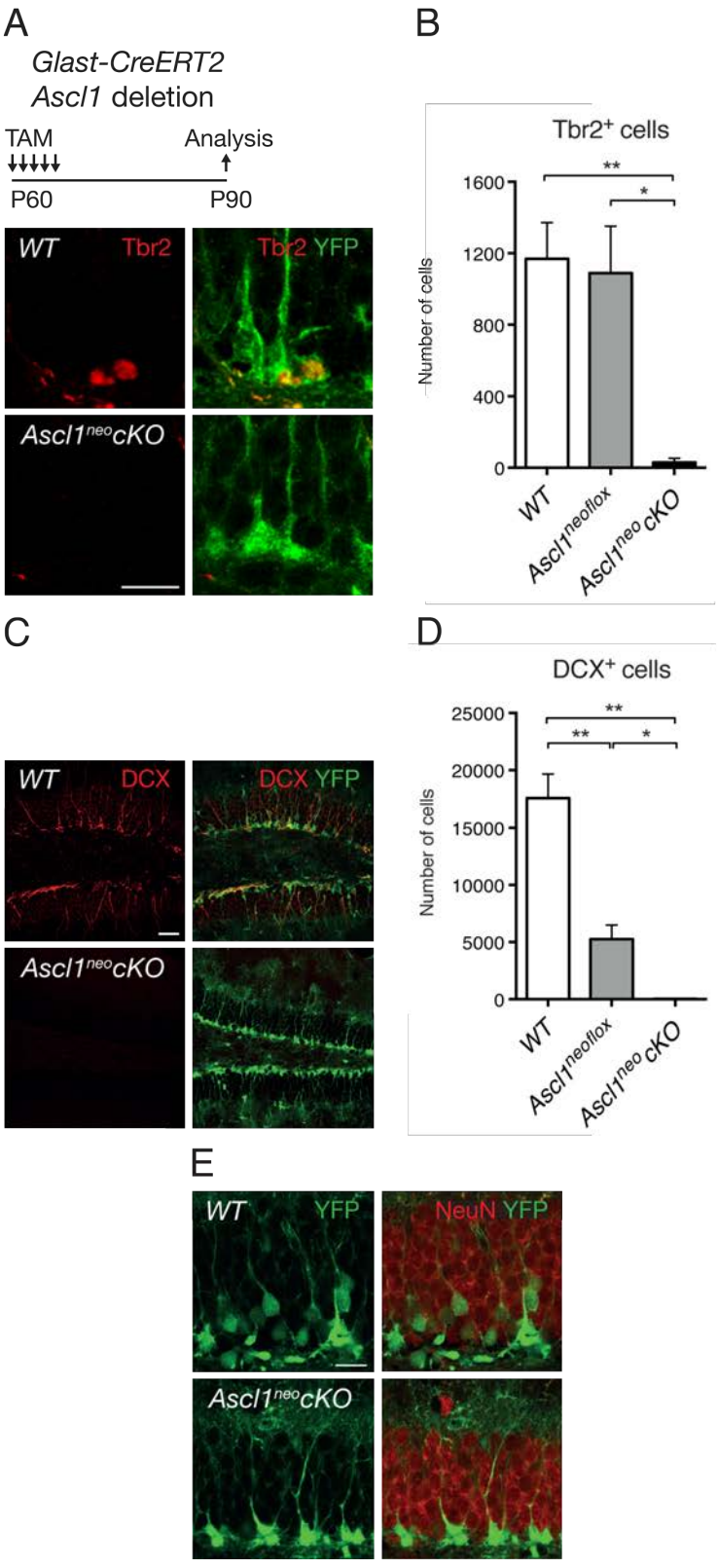

Figure 4-1 Block of neurogenesis after *Ascl1* deletion (legend next page)

Figure 4-1 Block of neurogenesis after *Ascl1* deletion

(A and B) Immunostaining for the IPC marker Tbr2 and YFP and quantification of the total number of $Tbr2^+$ YFP^+ cells in the DG show that IPCs are absent in $Ascl1^{neo}cKO$ mice. **(C and D)** Similarly, staining for the immature neuronal marker DCX and YFP and quantification of the total number of DCX^+ YFP^+ cells in the DG show that no new neurons are generated in $Ascl1^{neo}cKO$ mice. $Ascl1^{neoflox}$ mice, which have reduced levels of *Ascl1* expression, also show a reduction in the number of DCX^+ cells in the DG. **(E)** That no new neurons are born in $Ascl1^{neo}cKO$ mice can be observed by labelling for YFP and the mature neuronal marker NeuN. In *WT* animals, new-born YFP^+ neurons can be seen in the GL of the DG, and these express NeuN. No YFP^+ neurons can be found in in the GL of $Ascl1^{neo}cKO$ mice. n = 3 (B, D) for all genotypes. * $p<0.05$, ** $p<0.01$. Scale bars = 20µm (A, E) and 40µm (C).

Next, I performed immunostaining for the late IPC or immature neuron marker DCX, and after quantification I found these cells also to be lost after *Ascl1* deletion. In *WT* mice it is possible to find DCX^+ YFP^+ cells in the SGZ and GL of the DG, indicating that new cells were generated between TAM administration and analysis, while none of these cells are found in $Ascl1^{neo}cKO$ mice (Figures 4-1C and 4-1D). I also observed, differently from the Tbr2 data, a significantly lower number of DCX^+ cells in $Ascl1^{neoflox}$ animals, indicating that neuron generation is also affected by the level of *Ascl1* expression (*WT* versus $Ascl1^{neoflox}$ versus $Ascl1^{neo}cKO$, 17600 ± 2058 versus 5262 ± 1238 versus 0 cells; Figure 4-1D). Finally, both YFP expression in the GL and colocalisation with the mature neuronal marker NeuN confirm that no new neurons are generated and therefore neurogenesis is absolutely blocked after *Ascl1* is deleted (Figure 4-1E).

4.2 Effect of *Ascl1* deletion on astrogliogenesis

Stem cells in the DG of the hippocampus not only generate neurons, but are known to also give rise to astrocytes (Bonaguidi et al., 2011, Encinas et al., 2011). Two independent routes for generating astrocytes in the adult DG have been proposed. The first one involves astrogliogenic asymmetric RGL divisions. The second one, on the other hand, involves direct differentiation via transition astroglia or transition astrocytes (TA; Bonaguidi et al., 2011). Because GLAST is expressed not only by RGLs but also by mature astrocytes, it means that recombination using *Glast-CreERT2* animals occurs in both populations. This does not cause interference when studying the neurogenic lineage, since we find no expression of Ascl1 in astrocytes. However, by using this cre deleter line and following YFP^+ cells, I am unable to determine whether deletion of *Ascl1*, as well as blocking neurogenesis, affects the

generation of astrocytes. In order to circumvent this obstacle, I crossed *Ascl1*^{neoflox}; *R26-floxed stop-YFP* mice with mice carrying a *Nestin-CreERT2* allele instead of *Glast-CreERT2*. Nestin is expressed in RGLs, and recombination in astrocytes is not observed in animals carrying cre under this promoter (Encinas et al., 2011). In this way, by following the fate of YFP$^+$ cells one month after TAM administration I would be able to determine if astrocytes are generated after *Ascl1* loss (Figure 4-2A).

I first attempted to resolve this question by observing the morphology of YFP$^+$ cells in *WT* and *Ascl1*^{neo}*cKO* mice crossed with *Nestin-CreERT2* mice (see also Figures 4-3A and 4-3B for more on cellular phenotypes). Astrocytes exhibit numerous branching processes and are, consequently, often described as "bushy". I found no significant difference in the percentage of astrocyte-like cells among the total YFP$^+$ population between *WT* and *Ascl1*^{neo}*cKO* mice (*WT* versus *Ascl1*^{neo}*cKO*, 2.7% ± 1.7% versus 1.6% ± 1.4% astrocyte-like cells; Figure 4-2A). However, to discard the possibility of biasing the findings by focusing on morphology, and because the validity of this result depends on the total number of YFP$^+$ cells being equal between the two genotypes, I went on to specifically quantify those cells that were positive both for YFP and the mature astrocytic marker S100β. This analysis showed that, even though not significantly different, there are more double positive cells in *Ascl1*^{neo}*cKO* animals compared to *WT* (*WT* versus *Ascl1*^{neo}*cKO*, 274 ± 274 versus 3200 ± 1319 cells; Figure 4-2B). This analysis also revealed that while S100β$^+$ YFP$^+$ cells were all found in the CA region in *WT* mice, 30% of these cells in *Ascl1*^{neo}*cKO* animals were in the SGL/GL area of the DG, and they resembled the TAs that had been previously described by others (Bonaguidi et al., 2011; see also Figure 4-3B). This data might suggest that *Ascl1*-deficient RGLs are more likely to directly differentiate into astrocytes compared to *WT* RGLs. To address this, I quantified the number of TAs present in each of these genotypes using the *Glast-CreERT2* deleter line, which shows a more efficient recombination. I considered TAs those cells that were both GFAP and Nestin positive but that did not qualify as an RGL, namely, they were either in the GL as opposed to the SGZ, or had more than one process. This quantification did not convey a definite answer. There were more TAs in *Ascl1*^{neo}*cKO* mice compared to *WT* mice, but this was not a significant difference (*WT* versus *Ascl1*^{neo}*cKO*, 798 ± 99.1 versus 1105 ± 107 cells; Figure 4-

2C). Altogether, these results, even though not conclusive, point to a small increase in the direct differentiation of RGLs to astrocytes.

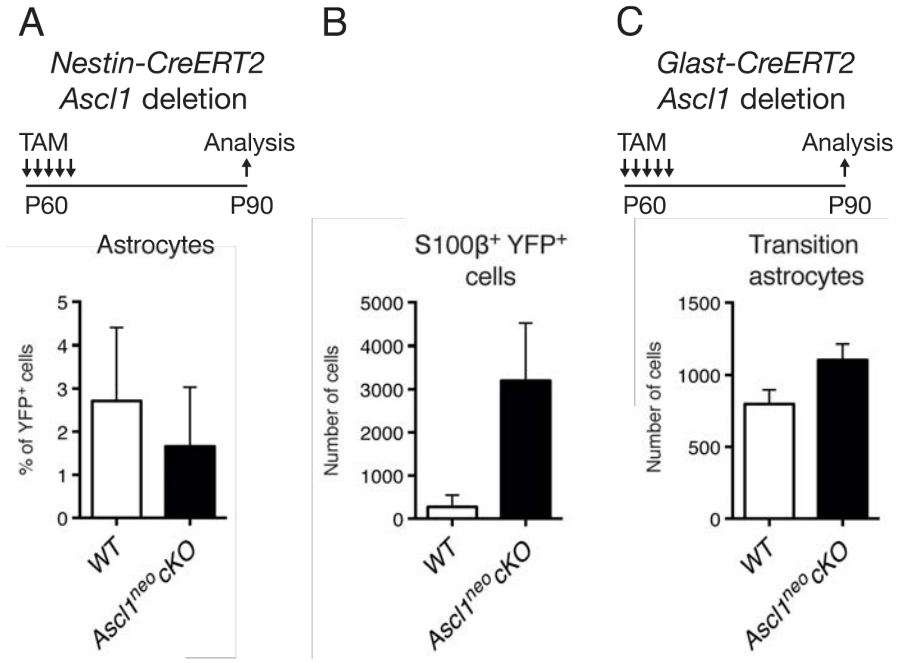

Figure 4-2 Effect of *Ascl1* deletion on astrogliogenesis

(A) Deletion of *Ascl1* using a *Nestin-CreERT2* deleter to study the fate of RGLs one month after TAM administration. Quantification of the percentage of YFP^+ cells showing astrocytic morphology (cell body with numerous branching processes) among all YFP^+ cells shows no apparent difference in the generation of astrocytes between *WT* and *Ascl1^{neo}cKO* mice. **(B)** Quantification of the total number of YFP^+ cells that are also $S100\beta^+$ in the hippocampus of *WT* and *Ascl1^{neo}cKO* mice shows that more astrocytes are found in *Ascl1^{neo}cKO* animals. Whether these are generated by direct differentiation from RGLs or through a proliferation step we cannot ascertain with this data. However, having in mind the complete lack of proliferation in *Ascl1^{neo}cKO* mice it appears likely that a small proportion of stem cells terminally differentiates into an astrocytic fate without going through a proliferative step. **(C)** Quantification of the total number of transition astrocytes (TAs, $GFAP^+$ $Nestin^+$ non-RGL) in the DG of *WT* and *Ascl1^{neo}cKO* animals shows a slight, but not significant, increase in TAs in *Ascl1*-deficient mice. n = 5 (A), 7 (B) and 2 (C) for *WT*; 7 (A), 9 (B) and 2 (C) for *Ascl1^{neo}cKO*.

4.3 Stem cells retain their characteristics after *Ascl1* deletion

4.3.1 RGLs are maintained after *Ascl1* deletion

Results so far show that neurogenesis is blocked after Ascl1 deletion. What happens, however, to RGLs? Can they maintain their characteristic properties after losing their ability to self-renew and generate new progeny? To answer these questions I first made use of *Nestin-CreERT2* animals again crossed with our *Ascl1^{neoflox}* mice.

Recombination efficiency in this cre line is very much reduced when compared to that of *Glast-CreERT2*, which makes it easier to follow cells and rely on morphology for analysis (Figures 4-3A and 4-3B). Moreover, quantification of RGL's progeny one month after *Ascl1* deletion showed results consistent with those previously obtained with the *Glast-CreERT2* line (Figure 4-3C). I found a proportion of YFP^+ cells in *WT* mice that resemble type 2 cells (or IPCs, 13.71% ± 5.53%), with the majority of them being $MCM2^+$ (57.65% ± 21.85%). No cells exhibiting type 2 morphology were found in *Ascl1^{neo}cKO* mice. Similarly, a big proportion of the YFP^+ cells present one month after recombination in *WT* mice had already differentiated into immature or mature neurons (40.32% ± 9.00%), as opposed to a small proportion of them in *Ascl1^{neo}cKO* mice (6.66% ± 2.60%). These cells most likely represent events of uncoupled recombination (where the *RYFP* locus undergoes recombination of at least one of its alleles, but *Ascl1* does not, see section 3.3.3), since I do not find such population of YFP^+ neurons using the *Glast-CreERT2* deleter line.

Next, I moved on to quantify the proportion of RGLs that are maintained one month after TAM administration. This analysis revealed that, in *Ascl1^{neo}cKO* mice, a majority of the cells that had undergone recombination had the characteristic radial morphology and had therefore been maintained as RGLs (35.59% ± 3.96% in *WT* and 76.48% ± 6.69% in *Ascl1^{neo}cKO*; Figures 4-3B and 4-3C). All together, these results suggest that *Ascl1* deletion leads to a block of neurogenesis but a maintenance of RGLs.

4.3.2 RGLs do not differentiate into the astrocytic lineage

Evidence so far suggests that stem cells are maintained despite their failure to divide, and does not point towards their aberrant differentiation. I, nonetheless, investigated the possibility of RGLs terminally differentiating into astrocytes. Analysis with the low-recombination *Nestin-CreERT2* mice already suggests that this might be the case for a very small proportion of the RGLs, but further quantification of YFP^+ $S100\beta^+$ cells in the DG of *Glast-CreERT2 Ascl1 WT* and *Ascl1^{neo}cKO* mice shows that the number of double positive cells remains the same (*WT* versus *Ascl1^{neo}cKO*, 974 ± 361 versus 1110 ± 173 cells; Figures 4-4A and 4-4B). Therefore, despite the

possibility of an increase in the number of TAs in the DG of *Ascl1*^{neo}*cKO* mice there is not an increase in the number of S100β^{+} cells, and therefore provide more evidence that RGLs retain their stem cell characteristics after *Ascl1* deletion.

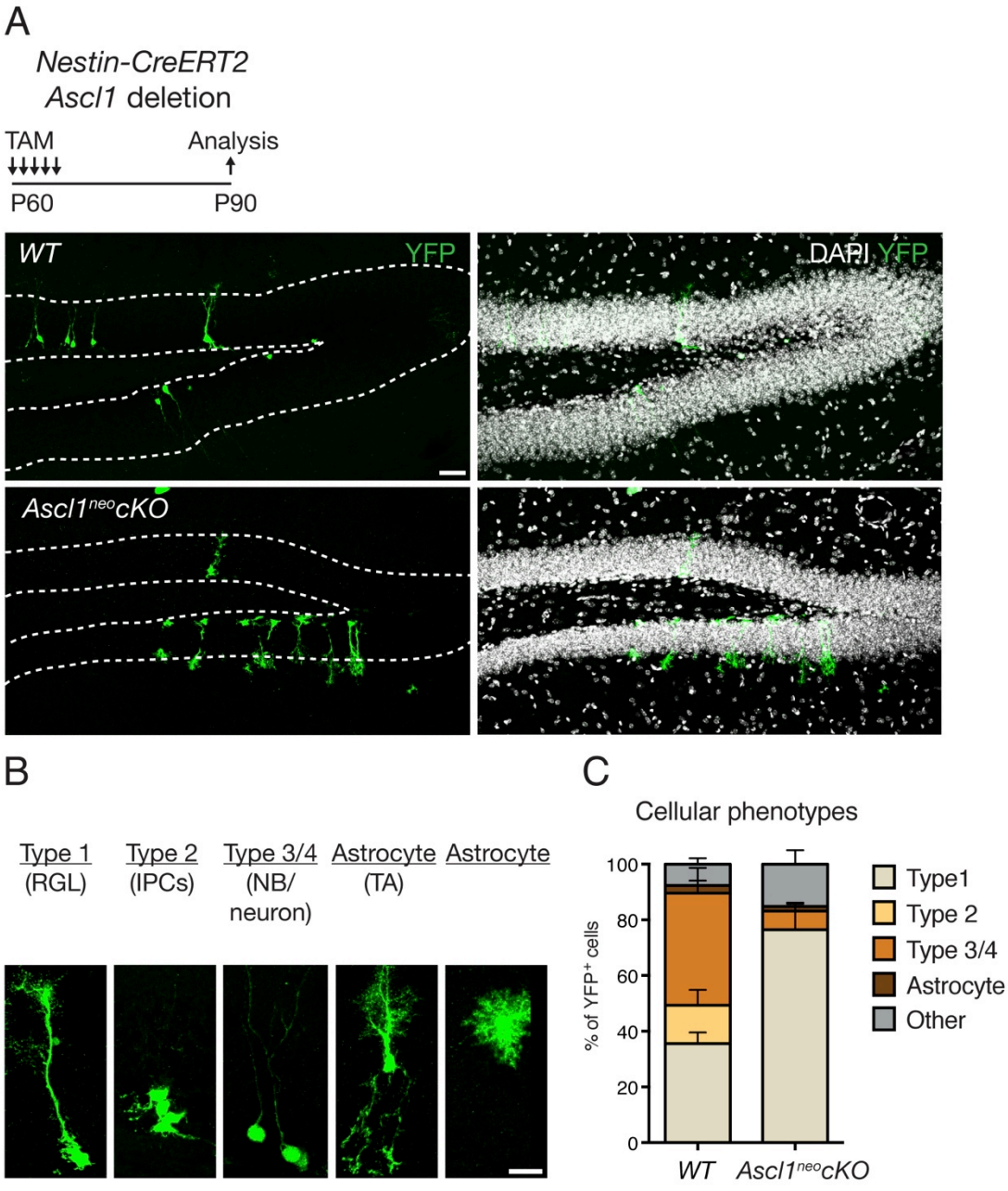

Figure 4-3 *Ascl1*-deficient cells retain their RGL morphology and are maintained in the adult DG (legend next page)

Figure 4-3 *Ascl1*-deficient cells retain their RGL morphology and are maintained in the adult DG

(A) The *Nestin-CreERT2* deleter line was crossed to *Ascl1*^{neoflox} mice to study the fate of *Ascl1*-deleted cells. TAM was administered at P60 and morphology of YFP^{+} (recombined) cells in the DG (and CA in the case of astrocytes) of both *WT* and *Ascl1*^{neo}*cKO* mice was examined one month later. Note the limited recombination efficiency obtained using the *Nestin-CreERT2* deleter line, which eases the study of cellular morphology. **(B)** Examples of the different type of cells recognised in the DG neurogenic lineage. Type 1 cells (RGLs) have their cell body in the SGZ and they have a radial process that extends along the GL. Type 2 cells (IPCs, three of them shown here) have small cell bodies positioned around the SGZ region and they possess small horizontal processes. Type 3/4 cells represent all neuroblasts (NB) and neurons. They have round cell bodies in the GL or SGZ and they have long thin processes extending towards the CA region. Two types of astrocyte-like cell types are recognised. Cells that resemble RGLs (but cannot be considered RGLs for not being positioned in the SGZ or having multiple processes) are called here transition astrocytes (TA), and highly ramified or "bushy" cells I called astrocytes. Characterisation of the cellular phenotype was assisted by immunostaining for the stem cell marker GFAP (not shown). **(C)** Quantification of the percentage of each of these cells found in *WT* and *Ascl1*^{neo}*cKO* mice among all YFP^{+} cells confirms that no new neurons are generated from cells that have lost *Ascl1*, and also shows that RGLs are maintained in this instance. The percentage of type 1 cells that remain one month after recombination is doubled in *Ascl1*^{neo}*cKO* mice compared to *WT*. A proportion of type 2 cells is found in *WT* but not *Ascl1*^{neo}*cKO* mice. Also, a big proportion of type 3/4 cells are found in *WT* animals, and only few of them are found in *Ascl1*-deficient mice. These type 3/4 cells in *Ascl1*^{neo}*cKO* animals likely represent uncoupled recombination events. There is no difference in the percentage of astrocytes found. TA astrocytes are included in the "other" category, which also includes any cell that did not fit into the rest of categories. n = 5 for *WT* and 7 for *Ascl1*^{neo}*cKO*. Scale bars = 20µm (B), 40µm (A).

During embryonic development, activation of Notch signalling has been reported to induce gliogenesis (Morrison et al., 2000, Wang and Barres, 2000). Therefore, one possibility for why RGLs are maintained in favour of terminal differentiation towards the astrogliogenic lineage, which could be expected in the absence of proliferation, is that there are reduced levels of Notch in RGLs. Notch ligands presented by IPCs (Lavado and Oliver, 2014) are indeed absent in this scenario, since IPCs are all depleted one month after *Ascl1* deletion. In order to determine if lower Notch activity might be responsible for the maintenance of RGLs, we measured expression of the Notch effectors *Hes1* and *Hes5* in laser capture-microdissected SGZ tissue. We found Notch activity to be indeed much reduced in *Ascl1*^{neo}*cKO* animals (Figure 4-4C), albeit not completely absent. The remaining Notch activity could be provided by other ligand-presenting cells (e.g. blood vessel endothelial cells), although this remains to be determined. These results point to a possible mechanism for the block of astrocytic differentiation of RGLs in *Ascl1*^{neo}*cKO* animals.

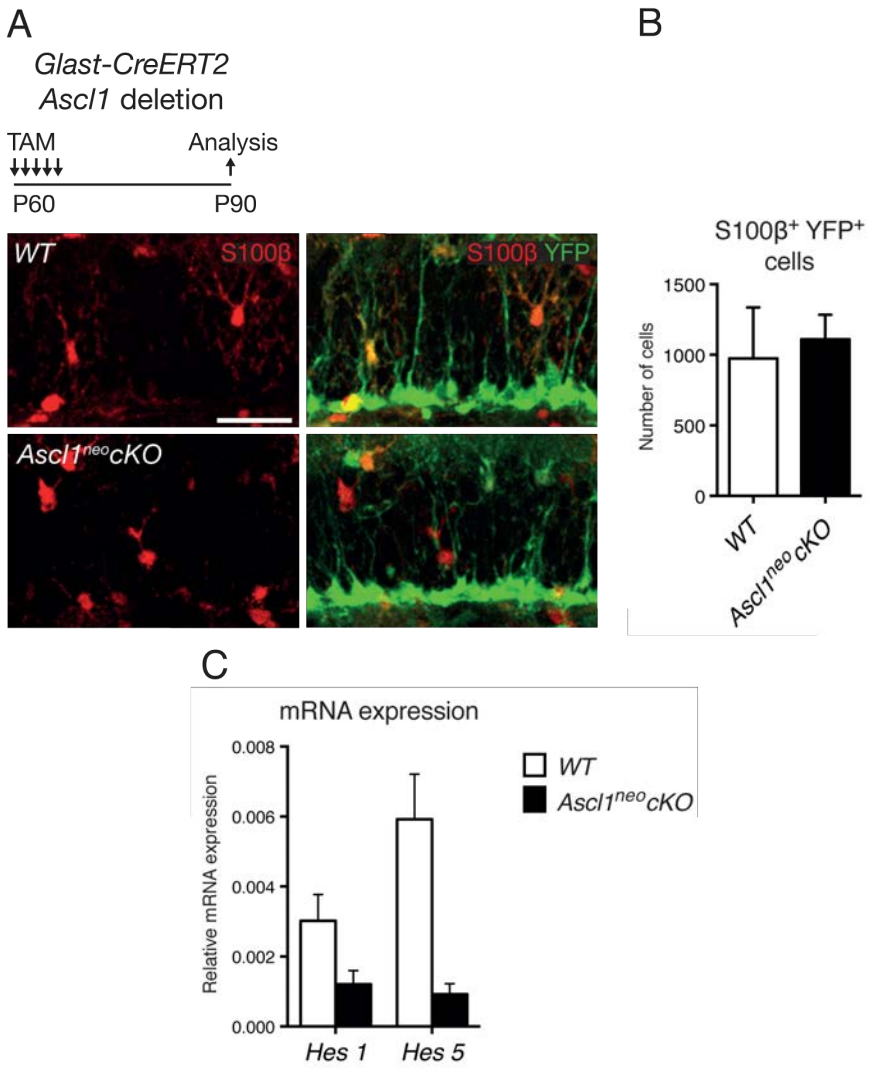

Figure 4-4 Stem cells do not express markers of mature astrocytes

(A and B) Labelling for the astrocytic mature marker S100β and YFP and quantification of the total number of S100β$^+$ YFP$^+$ cells shows that there is no difference in the number of astrocytes in the DG between *WT* and *Ascl1^{neo}cKO* mice, and that therefore RGLs do not express mature astrocytic markers and retain their undifferentiated stem cell character. **(C)** Quantitative RT-PCR analysis of LCM tissue for the Notch transcriptional effectors *Hes1* and *Hes5* show that Notch signalling activity is reduced in the SGZ of *Ascl1^{neo}cKO* mice. Expression levels are normalized to *Gapdh*. (C) Performed by Ayako Ito. n = 2 (B) and 4 (C) for both genotypes. Scale bar = 40μm (A).

4.3.3 RGLs do not differentiate into the oligodendrocytic lineage

Next, we went on to confirm *Ascl1*-deficient cells do not take on an aberrant fate and differentiate into cells of the oligodendrocytic lineage. Retrovirus-mediated overexpression of *Ascl1* in the DG has been shown to result in the exclusive generation of cells of the oligodendrocytic lineage (Jessberger et al., 2008).

Quantification of YFP^+ $Olig2^+$ cells one month after TAM administration revealed that: 1) very few oligodendrocyte cells are generated in the DG, and that 2) there was no difference in the number of those generated between *WT* and *Ascl1^{neo}cKO* mice (*WT* versus *Ascl1^{neo}cKO*, 192 ± 192 versus 128 ± 64 cells; Figures 4-5A and 4-5B). These results suggest that no aberrant differentiation of RGLs after *Ascl1* deletion is taking place.

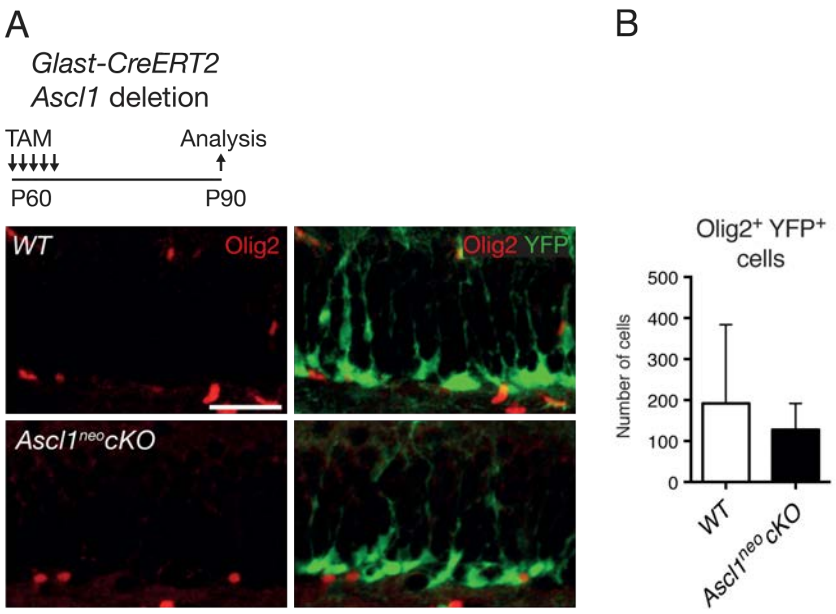

Figure 4-5 Stem cells do not aberrantly differentiate into the oligodendrocytic lineage

(A and B) Labelling for the oligodendrocyte marker Olig2 and YFP and quantification of the total number of $Olig2^+$ YFP^+ cells shows that there are very few oligodendrocytes generated in the DG of adult mice and that there is no difference in this number between *WT* and *Ascl1^{neo}cKO* mice. n = 3 (B). Scale bar = 40µm (A).

4.3.4 RGLs do not become senescent

Ascl1-deleted RGLs are in a quiescence-like state, since I have shown they do not produce progeny and do not aberrantly differentiate into other lineages. Another possibility to explore was that of these cells having become senescent. Senescence is a permanent retirement out of the cell cycle, and it is generally induced by stimuli that put a cell at risk, the most common being DNA damage (Blomen and Boonstra, 2007). *Ascl1* deletion could be driving RGLs into a non-proliferative senescent state. To test this possibility we performed expression analysis by RT-qPCR for the

senescence-associated cyclin-dependent kinase inhibitor $p16^{INK4a}$ (Molofsky et al., 2006) in SGZ tissue obtained with LCM. Both *WT* and $Ascl1^{neo}cKO$ mice show very low levels for $p16^{INK4a}$, and there is no difference in expression between the two genotypes (Figure 4-6), thus suggesting that RGLs that have deleted *Ascl1* are not senescent.

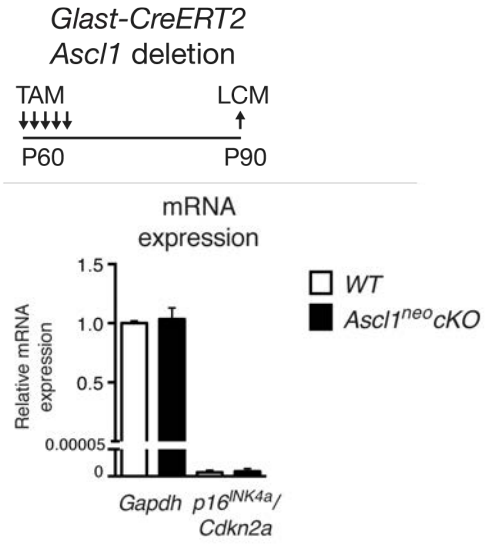

Figure 4-6 *Ascl1*-deficient cells do not become senescent

Quantitative RT-PCR analysis for the cyclin-dependent kinase inhibitor $p16^{INK4a}$/*Cdkn2a* in LCM SGZ tissue shows that expression of this marker is scarcely detected in the DG of P90 mice and it is not increased in $Ascl1^{neo}cKO$ animals compared to *WT* animals. Expression levels are normalized to *Gapdh* (also shown here as a scale). Performed by Ayako Ito. n = 3 for both genotypes.

4.3.5 RGLs retain stem cell characteristics

Finally, I performed immunohistochemical analysis of known stem cell markers to verify that RGL characteristics are indeed maintained in *Ascl1*-deleted animals. As mentioned in the previous section, RGLs in these mice retain their characteristic radial morphology. Importantly, the radial processes in these cells express both GFAP and Nestin (Figure 4-7A). In the case of *Ascl1*-deficient cells, the level of Nestin expression was in occasion lower or barely detectable when compared to *WT* cells, and this is translated into a significant lower proportion of $GFAP^+$ cells that express Nestin in $Ascl1^{neo}cKO$ animals (Figure 4-7B). This is consistent with reports indicating that quiescent cells express lower levels or no Nestin (Codega et al., 2014, DeCarolis et al., 2013).

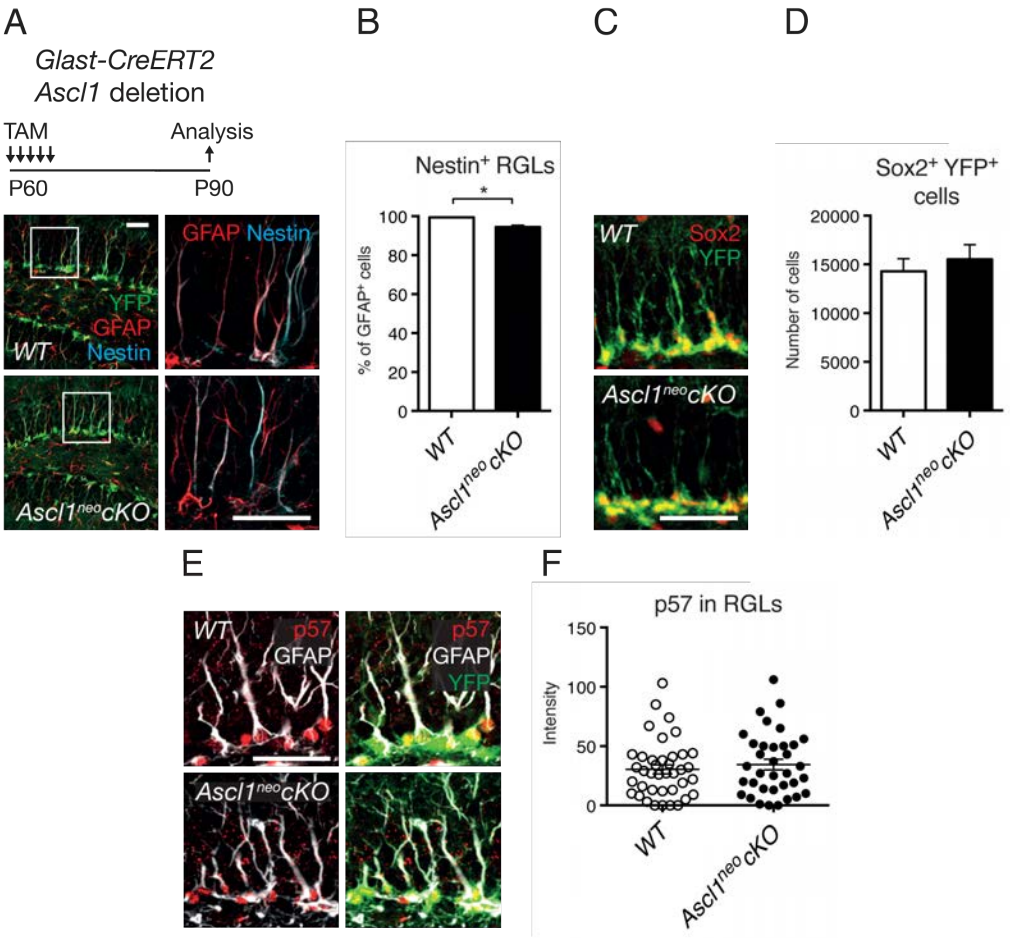

Figure 4-7 RGLs retain stem cell characteristics after *Ascl1* deletion

(A and B) Administration of TAM at P60 and immunostaining analysis one month later reveals that *Ascl1*-deficient RGLs still express GFAP and Nestin, two markers characteristic of the stem cell phenotype. Quantification of the percentage GFAP radial cells that are also Nestin^{+} shows that in *WT* animals almost all RGLs express both markers. In *Ascl1^{neo}cKO* animals, the vast majority of GFAP^{+} cells also express Nestin. The level of expression in these animals, however, is slightly lower than that in *WT* mice (not shown), and this is reflected in a significant difference in the percentage of cells expressing both markers between them. **(C and D)** Immunohistochemistry for YFP and the precursor marker Sox2 shows that *Ascl1*-deleted RGLs retain expression of this marker, and quantification of the total number of Sox2^{+} YFP^{+} cells in the DG shows no difference between *WT* and *Ascl1^{neo}cKO* animals. **(E)** *Ascl1*-deficient RGLs also retain the expression of the quiescence marker p57, shown by immunolabeling for p57, GFAP and YFP. **(F)** Quantification of the intensity of the nuclear p57 staining in RGLs in *WT* and *Ascl1^{neo}cKO* animals shows that stem cells that have lost *Ascl1* retain levels of this marker that are comparable to those levels in *WT* cells. n = 2 (F) and 3 (B, D) for *WT*; 2 (B, D, F) for *Ascl1^{neo}cKO*. * $p<0.05$. Scale bars = 40µm (A, C and E).

In a similar manner, I did not find any difference in the expression of the stem cell marker Sox2. The number of YFP^{+} Sox2^{+} cells was comparable in *WT* and *Ascl1^{neo}cKO* mice, with all RGLs expressing this marker (Figures 4-7C and 4-7D). The cyclin-dependent kinase inhibitor p57 has been described to be expressed in

RGLs and to contribute to the regulation of RGL quiescence (Furutachi et al., 2013). Immunolabeling for p57 showed that *Ascl1*-deficient RGLs do not lose the expression of this marker (Figure 4-7E), and that the intensity of its expression is comparable to that of *WT* mice (Figure 4-7F). Therefore, *Ascl1* loss does not disrupt stem cell maintenance or quiescence.

4.4 Long-term effect of *Ascl1* deletion

4.4.1 RGLs remain quiescent long-term

Stem cells in *Ascl1^{neo}cKO* mice appear to be quiescent one month after *Ascl1* deletion. I next went on to examine whether this state was stable over a longer period of time. For this, two-month old mice received TAM over five consecutive days as before and analysis was carried out 5 months after at P210 (Figure 4-8). In *WT* mice we can observe the number of GL-positioned YFP$^+$ neurons that were generated over this period (Figure 4-8, upper panel). We can also find a proportion of RGLs that is Ki67$^+$ and therefore dividing (Figure 4-8, upper inset panel). In *Ascl1^{neo}cKO* mice, on the other hand, we can see that no new neurons were generated during this time (Figure 4-8, bottom panel). Nevertheless, RGLs still retained their characteristic radial morphology and expressed stem cell markers like GFAP, Nestin and Sox2 (Figure 4-8 and not shown). These cells were also negative for the proliferation marker Ki67$^+$ (Figure 4-8A, bottom inset panel), indicating that *Ascl1*-deficient RGLs remain inactive and quiescent long-term.

Figure 4-8 *Ascl1*-deficient RGLs remain non-proliferative long-term

Administration of TAM over five consecutive days and analysis five months later to study the effect of *Ascl1* deletion long-term. Immunolabeling for YFP shows that, while in *WT* animals a big number of neurons were generated in this time (rounded YFP$^+$ cells in GL), no new neurons were generated in *Ascl1^{neo}cKO* mice. In addition, staining for Ki67 and GFAP show that stem cells in the DG of *Ascl1^{neo}cKO* mice not only remain non-proliferative (Ki67$^-$), but they also retain their characteristic radial morphology. RGLs in *WT* animals still retain their proliferative ability (see Ki67$^+$ GFAP$^+$ YFP$^+$ RGL in inset, white square). Scale bars = 40µm and 10µm in enlargement.

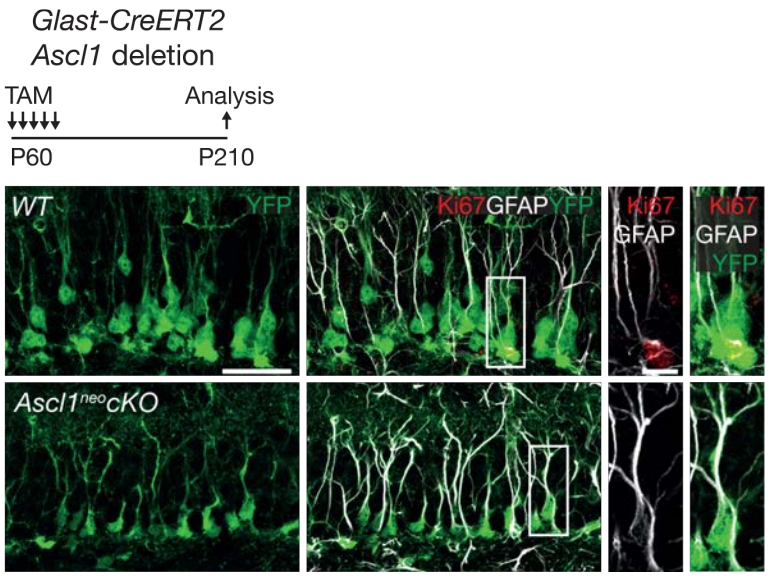

Figure 4-8 *Ascl1*-deficient RGLs remain non-proliferative long-term (legend previous page)

4.4.2 *Ascl1* deletion prevents RGL exhaustion

Hippocampal neurogenesis is reduced with age, and this reduction is attributed, in part, to non-self-renewing RGL divisions (Bonaguidi et al., 2012, Encinas and Sierra, 2012; see also section 1.3.4.1). Quantification of the total number of stem cells over time in *WT* animals showed that indeed, this number significantly decreases between P64 and P90 and that it decreases further at P210 (Figure 4-9). The same quantification in *Ascl1^{neo}cKO* mice showed, however, that the total number of RGLs does not change between P64 and P90, and only slightly but not significantly decreases at P210 (*WT* versus *Ascl1^{neo}cKO*, 22116 ± 1681 versus 24533 ± 1399 RGLs at P64; 14453 ± 1021 versus 26098 ± 1913 RGLs at P90; 12516 ± 1603 versus 20764 ± 395 at P210; Figure 4-9). This data supports the finding that RGLs in *Ascl1^{neo}cKO* mice do not divide, and suggests that *Ascl1* deletion prevents the attrition of the RGL pool.

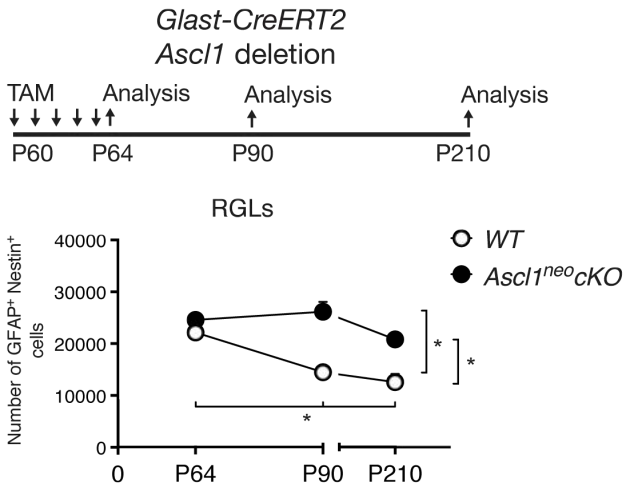

Figure 4-9 Hippocampal stem cell attrition is prevented by *Ascl1* deletion

Quantification of the total number of stem cells ($GFAP^+$ $Nestin^+$ radial cells) at P64, P90 and P210 in *WT* and *Ascl1^{neo}cKO* mice that received TAM at P60. *WT* animals show a reduction in total stem cell numbers from P64 to P90 and a further decrease at P210. In *Ascl1^{neo}cKO* mice, the number of stem cells remains constant from P64 to P90, and only slightly and not significantly decreases at P210. n = 3 for both genotypes. * $p<0.05$.

Chapter 5 Results

Ascl1 expression and neurogenic stimuli

Deletion of *Ascl1* results in a block of neurogenesis due to the inability of stem cells to enter the cell cycle and proliferate. The role of RGLs in the adult hippocampus is that of sensors, in that they need to be able to sense and integrate the different stimuli present in the environment to then generate appropriate responses. In this way stem cells in the DG mediate the response to changing physiological demands, as well as to pathological insults. In this chapter I will be exploring the response of Ascl1 to neurogenic and anti-neurogenic stimuli. Not much is known at the moment as to how RGLs are able to respond to their environment in response to stimuli. The expression of Ascl1 and what we know of its function so far highlight it as a possible candidate to fulfil this role.

5.1 Ascl1 expression is regulated in response to neurogenic and anti-neurogenic stimuli

5.1.1 Voluntary running does not affect stem cell activity

I started by examining the effect of neurogenic stimuli on stem cell activity and Ascl1 expression. If *Ascl1* is to be an important factor in the response to stimuli, then its expression should be upregulated in RGLs upon their activation. Exercise is a potent neurogenic stimulus. Its impact on RGL activity has been much debated. Some groups have reported an effect of running on the stem cell population (Lugert et al., 2010), while others claim that this effect is restricted to the IPC population (Kronenberg et al., 2003). Exposure to a running wheel for 12 days resulted in an overall increase of proliferation in *WT* mice (Figure 5-1). Quantification of the proportion of $GFAP^+$ radial cells that express MCM2 showed, however, that, at least with the procedure that we followed, RGL activity is not affected by voluntary running (Control versus RUN, 4.4% ± 0.5% versus 3.2% ± 1.0% $MCM2^+$ RGLs; Figure 5-2A). Correspondingly, Ascl1 expression is unchanged in RGLs after 12 days of voluntary exercise (Control versus RUN, 1.4% ± 0.3% versus 1.3% ± 0.6% $MCM2^+$ RGLs; Figure 5-2B).

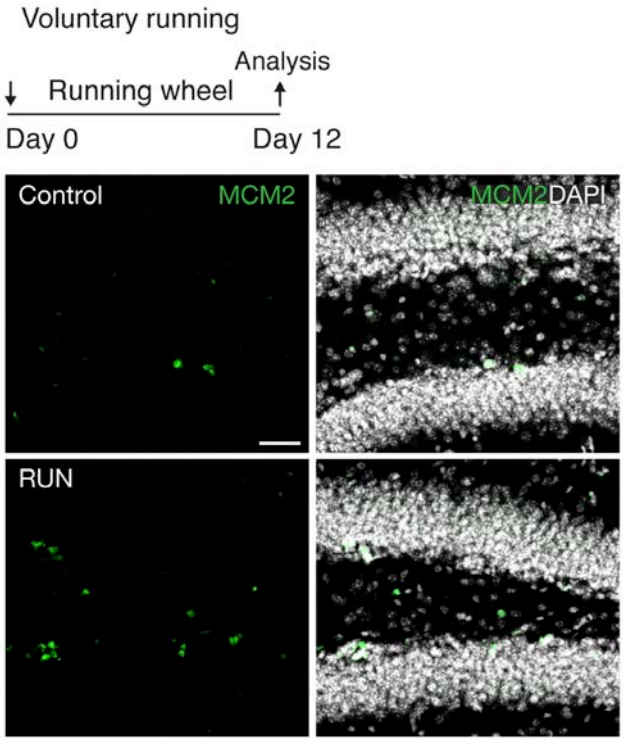

Figure 5-1 Voluntary running increases total proliferation in the DG

Voluntary running for 12 days in adult WT mice increases the number of active cells in the DG, illustrated here by immunostaining for MCM2. n = 4. Scale bar = 40µm

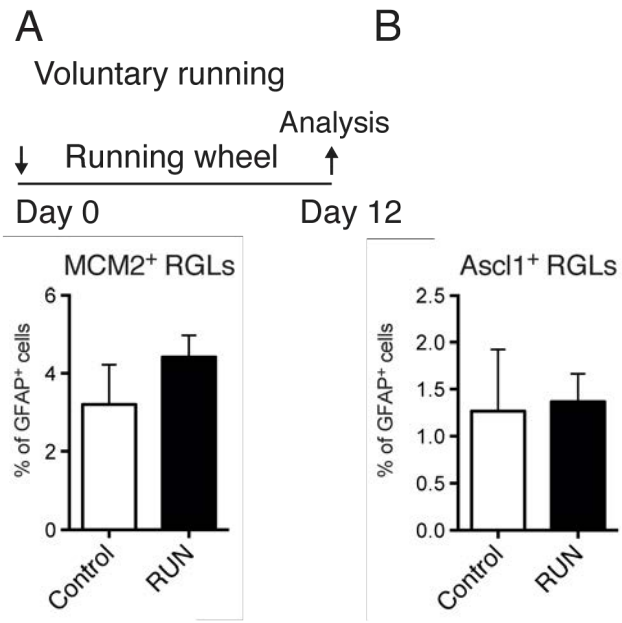

Figure 5-2 Voluntary running does not affect hippocampal stem cell activation (legend next page)

Figure 5-2 Voluntary running does not affect hippocampal stem cell activation

(A) Quantification of the percentage of $GFAP^+$ radial stem cells that are positive for MCM2 revealed that the increase of $MCM2^+$ cells does not correspond with an increase in activated RGLs; there is no difference in the proportion of $MCM2^+$ RGLs between control animals and runners. **(C)** Similarly, no difference in the percentage of $Ascl1^+$ RGLs is observed between control and RUN animals. n = 4 (B) and 6 (C) for control; 7 (B) and 9 (C) for RUN.

5.1.2 Social isolation negatively regulates Ascl1 expression

Another known stimulus that regulates stem cell activity is acute stress (Dong et al., 2004). Social isolation and transfer of mice from a grouped-cage to a singled-cage environment elicits a stress response in mice, and consequently negatively regulates stem cell activity. Isolation of two-month-old *WT* animals and analysis one or two days later revealed that this negative stimulus modulates Ascl1 expression in RGLs (Ctrl 0 versus Isol D1 versus Isol D2, 3.4% ± 0.9% versus 4.5% ± 0.4% versus 1.8% ± 0.8% $Ascl1^+$ RGLs; Figure 5-3B). A reduction in the number of $Ascl1^+$ RGLs two days after isolation is translated into a slight decrease in the number of $MCM2^+$ RGLs (Ctrl 0 versus Isol D1 versus Isol D2, 4.5% ± 1.9% versus 6.2 ± 1.1% versus 3.9% ± 0.9% $MCM2^+$ RGLs; Figure 5-3A). Ascl1 expression is, therefore, modulated in RGLs upon the reception of anti-neurogenic stimuli.

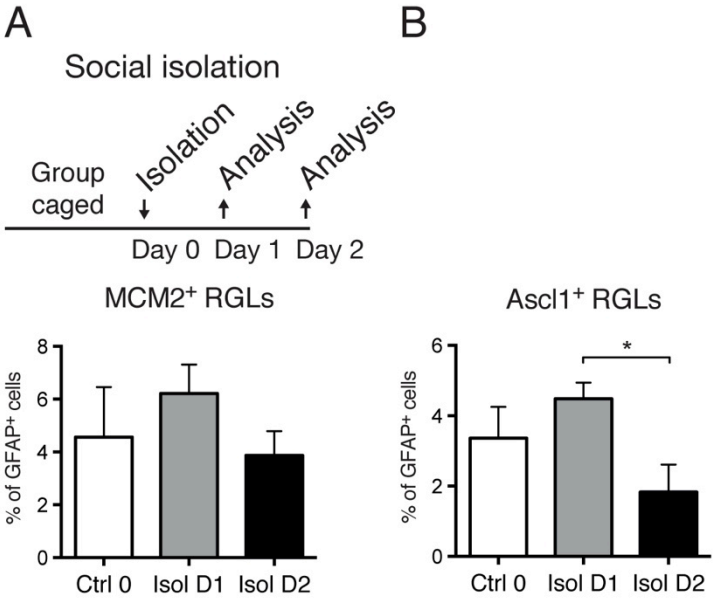

Figure 5-3 Social isolation is an anti-neurogenic stimulus that negatively regulates Ascl1 in RGLs (legend next page)

Figure 5-3 Social isolation is an anti-neurogenic stimulus that negatively regulates Ascl1 in RGLs

(A) Social isolation paradigm to test the effect of an anti-neurogenic stimulus on Ascl1 expression and stem cell activity. Before the start of the experiment *WT* mice were kept in a large cage with 4-8 littermates (Group caged). On day 0, animals were placed on small cages on their own (Ctrl 0) and analysis was carried out one or two days later (Isol D1 or Isol D2, respectively) by performing immunohistochemistry for MCM2 and Ascl1. Quantification of the percentage of MCM2-expressing $GFAP^+$ radial cells shows that stem cell activation is slightly reduced on the second day of isolation (Isol D2). **(B)** The reduction in $MCM2^+$ RGLs at Isol D2 corresponds with a significant decrease in the percentage of $Ascl1^+$ RGLs. n = 4 (A, B) for Isol D1and Isol D2; 5 for Ctrl 0. * $p<0.05$.

5.1.3 Ascl1 is upregulated after removal of an anti-neurogenic stimulus

I next made use of a genetic approach to further study Ascl1 regulation in RGLs of the DG after the removal of an anti-neurogenic stimulus. Notch signalling is an important regulator of stem cell maintenance, and deletion of the Notch pathway component *RBPJκ* induces the activation of RGLs (Ehm et al., 2010). TAM administration in 3 month-old mice carrying an inducible *RBPJκ*-mutant allele as well as the *Glast-CreERT2* deleter and examination of stem cell activity 7 days later revealed a dramatic increase in the number of $MCM2^+$ RGLs as expected (*WT* versus *RBPJκ cKO*, 1.6% ± 0.6% versus 17.7 ± 3.4 $MCM2^+$ $Ascl1^-$ RGLs; Figures 5-4A and 5-4B). More strikingly, I saw a similarly dramatic induction of Ascl1 expression in RGLs after loss of Notch signalling compared to control mice. I also found an increased proportion of RGLs expressing Ascl1 but not MCM2, a population of cells that was almost negligible in normal conditions (*WT* versus *RBPJκ cKO*, 1.5% ± 0.7% versus 26.7% ± 1.1% $MCM2^+$ $Ascl1^+$ RGLs; 0.2% ± 0.2% versus 3.6% ± 1.5% $MCM2^-$ $Ascl1^+$ RGLs; Figure 5-4B).

The induction of Ascl1 was specific and not a general response of proneural factors to the loss of Notch, as shown by *in situ* hybridisation (ISH) for both *Ascl1* and *Neurog2* (Figures 5-5A and 5-5B). *Ascl1* is upregulated after *RBPJκ* deletion, but this is not the case for *Neurog2*, whose expression in the adult DG appears undetectable by ISH (see Figure 5-5C showing an ISH for *Neurog2* in the E14.5 mouse telencephalon as a control). Together, these data demonstrate that anti-neurogenic signals like Notch supress Ascl1 expression.

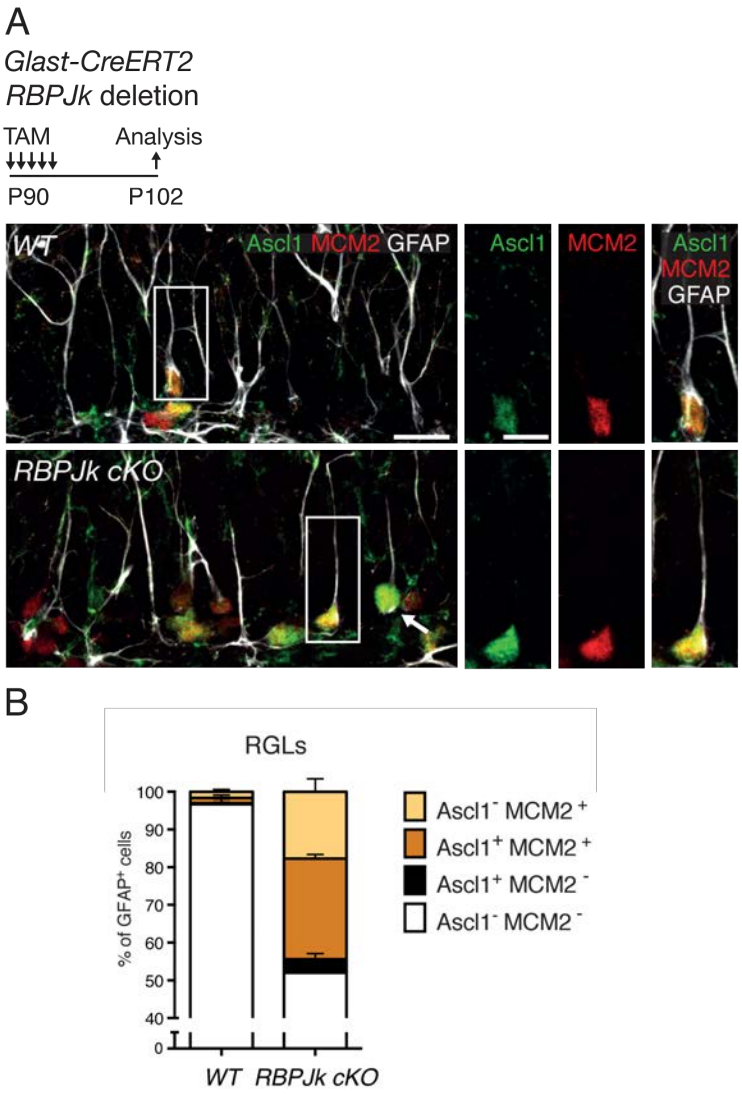

Figure 5-4 Ascl1 upregulation in hippocampal stem cells after the removal of an anti-neurogenic stimulus

(A and B) Conditional deletion of *RBPJκ* after TAM administration in P90 animals and analysis five days after the last injection. Labelling for Ascl1, MCM2 and GFAP and quantification of the percentage of $Ascl1^+$ and/or $MCM2^+$ $GFAP^+$ radial cells reveals that the loss of Notch signalling results in a dramatic activation of stem cells in the DG, with an increase in the proportion of $MCM2^+$ RGLs (light and dark orange bars, B; white arrow, A). This activation is accompanied by an increase in the percentage of $Ascl1^+$ RGLs (dark orange and black bars; white arrow, A), and by an increase of $Ascl1^+$ $MCM2^-$ stem cells (black bars). n = 5 for *WT*; 7 for *RBPJκ cKO*. p values, $MCM2^+$ RGLs in *WT* versus *RBPJκ cKO* < 0.0001; $Ascl1^+$ RGLs in *WT* versus *RBPJκ cKO* < 0.0001; $Ascl1^+$ $MCM2^-$ RGLs in *WT* versus *RBPJκ cKO* = 0.0911. Scale bars = 20μm in main panel and 10μm in enlarged panel.

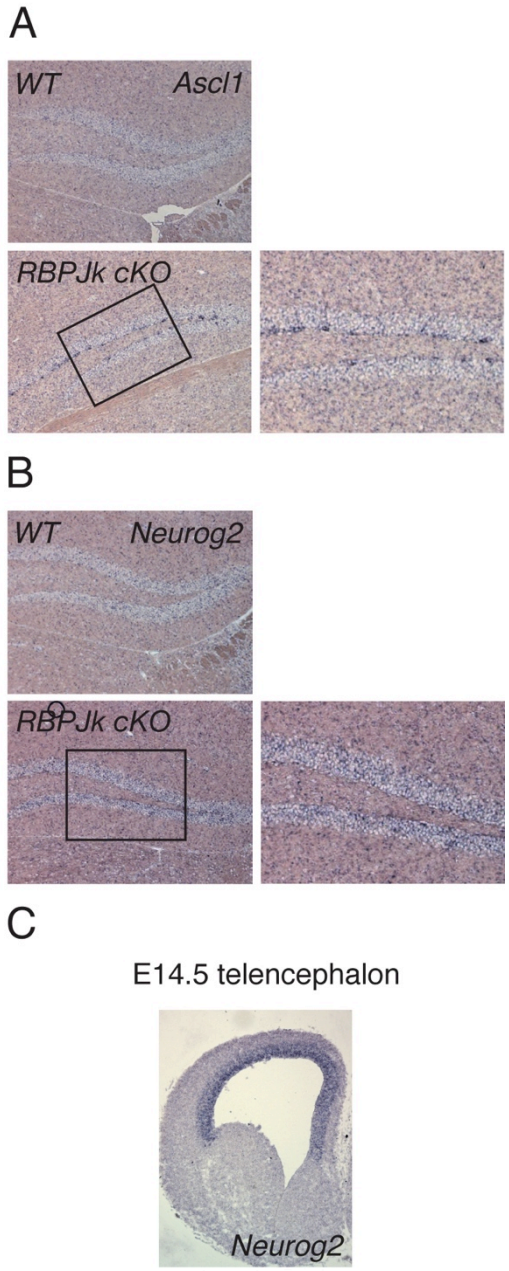

Figure 5-5 Ascl1 upregulation after Notch signalling inactivation is specific

(A) *In situ* hybridisation (ISH) for *Ascl1* shows that the upregulation of Ascl1 protein observed after *RBPJκ* deletion is, at least in part, due to an upregulation in *Ascl1* transcription. *RBPJκ cKO* mice, which received TAM for five days at P90 and were sacrificed for analysis seven days after, present a great increase of *Ascl1* signal in the SGZ (black box, inset) when compared to *WT* animals. **(B)** This upregulation of *Ascl1* transcription is not general for all proneural factors, but specific to *Ascl1*. ISH for *Neurog2* in *WT* and *RBPJκ cKO* mice shows no difference in *Neurog2* transcription in the DG, with very low or undetectable levels for both genotypes. **(C)** ISH for *Neurog2* in the E14.5 embryonic mouse brain as a positive control showing signal in the dorsal telencephalon.

5.1.4 Ascl1 is upregulated early in response to seizures

Finally, in order to study Ascl1 expression in response to a stimulus in more detail, I made use of kainic acid (KA), a potent pharmacological stimulus that is known to activate stem cells (Steiner et al., 2008). I used two month-old *WT* mice and administered a sub-seizure dose of KA (19 mg/kg) that was enough to have an effect on RGL activity (Figure 5-6A). Measuring stem cell activation by quantifying the proportion of $MCM2^+$ RGLs 1, 2, 4 and 7 days after KA injection, I found that by the second day there is an upregulation of the percentage of active RGLs when compared to animals that received saline. This increase becomes significant 4 days after KA administration (Saline versus KA, 4.1% ± 0.9% versus 3.4% ± 1.0% at Day 1; 2.0% ± 1.2% versus 9.6% ± 2.5% at Day 2; 2.7% ± 0.5% versus 13.9% ± 1.1% at Day 4; 4.2% ± 0.6% versus 7.1% ± 1.0% $MCM2^+$ RGLs; Figure 5-6B). The activation in response to KA appears to be a transient event, since 7 days after the injection the proportion of RGLs that was $MCM2^+$ was already reduced.

Interestingly, when examining the expression of Ascl1 in RGLs, I found that Ascl1 is upregulated already one day after KA administration (Saline versus KA, 3.7% ± 0.5% versus 8.6% ± 2.7% at Day 1; 0.5% ± 0.5% versus 6.4% ± 1.5% at Day 2; 1.6% ± 0.02% versus 6.4% ± 1.1% at Day 4; 3.4% ± 1.0% versus 3.5% ± 0.9% $Ascl1^+$ RGLs; Figure 5-6C), and the upregulation is maintained for 4 days, after which Ascl1 expression in RGLs returns to baseline levels. This data highlights that a significant fraction of RGLs that are positive for Ascl1 at 24 hours after injection are negative for MCM2 (Saline versus KA, 1.4% ± 0.9% versus 7.8% ± 1.3% at Day 1; 0% versus 0.4% ± 0.4% at Day 2; 0.5% ± 0.5% versus 0.6% ± 0.3% at Day 4; 0.4% ± 0.4% versus 0.6% ± 0.3% at Day 7; Figure 5-6D), and thus indicates that Ascl1 induction precedes RGL activation. Altogether, this data shows that neurogenic stimuli rapidly induce Ascl1 expression and subsequently quiescence exit in RGLs.

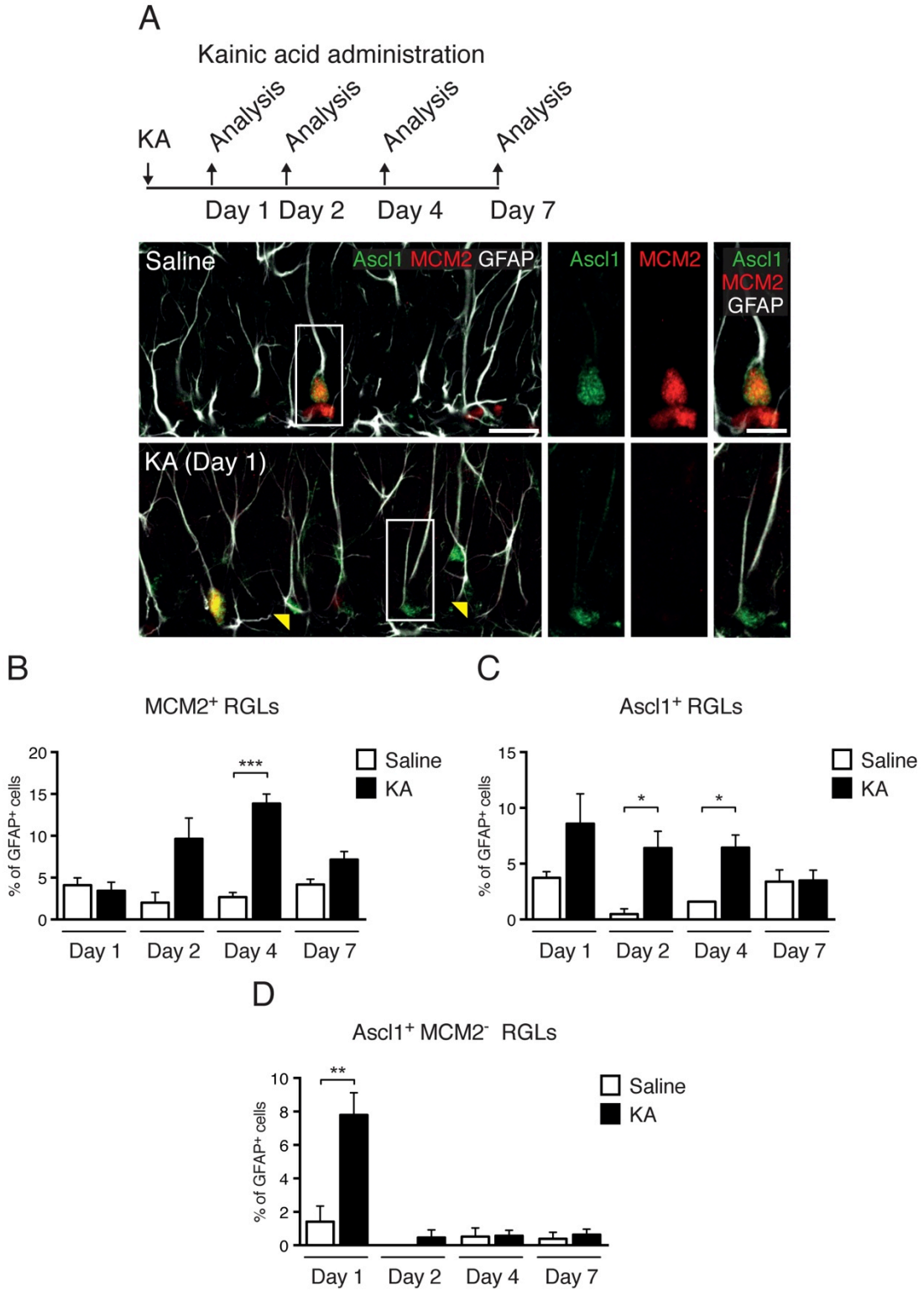

Figure 5-6 Early Ascl1 upregulation in response to neurogenic stimuli

(A) Administration of saline or kainic acid (KA) by i.p. injection to adult *WT* mice and analysis one (Day 1), two (Day 2), four (Day 4) or seven (Day 7) days later to study stem cell activity. Labelling for Ascl1, MCM2 and GFAP show that KA induces stem cell activation and Ascl1 upregulation from Day 1. **(B)** Quantification of the percentage of active RGLs ($MCM2^+$) among all $GFAP^+$ radial cells shows that KA induces MCM2 expression from Day 2. This induction is maintained at four days but is downregulated to levels almost comparable to saline by Day 7 (*legend continued in next page*).

(C) Quantification of Ascl1$^+$ RGLs in saline and KA-injected mice revealed that KA administration induces Ascl1 upregulation after only one day (yellow arrowheads and inset in white box, A), indicating that stem cells upregulate Ascl1 and only after express MCM2. Ascl1 upregulation in KA-injected mice is maintained up to Day 4, and by Day 7 its expression is back to baseline levels. **(D)** That Ascl1 is upregulated from Day 1 and MCM2 is not indicates that, as shown here as the percentage of Ascl1+ MCM2- RGLs, one day after KA injection there is a population of cells that express Ascl1 but do not express MCM2, showing that stem cells express Ascl1 and only after exit quiescence. n = 3 (B, C, D) for Saline Day 2, Day 4 and Day 7, and for KA Day 7; 4 (B, C, D) for Saline Day 1, and for KA Day 2 and Day 4; 5 (B, C, D) for KA Day 1. * p<0.05, ** p<0.01, *** p<0.001. Scale bars = 20µm in main panel and 10µm in enlarged panel.

5.2 *Ascl1*-deficient RGLs do not respond to neurogenic stimuli

The results above show that Ascl1 is regulated in RGLs in response to stimuli. The next question to ask was whether this regulation was required for the response of RGLs to the stimuli. To answer this question I made use of *Ascl1*^{neoflox} animals to delete *Ascl1* at P60 and investigate their response to KA after 4 days of injection (see Figure 5-7A for injection timings). In *WT* animals KA strongly induced neurogenesis, and this is shown by the quantification of the number of MCM2$^+$ cells (Figures 5-7A and 5-7B). In contrast, KA entirely failed to activate RGLs in *Ascl1*^{neo}*cKO* mice and to consequently induce neurogenesis (KA *WT* versus *Ascl1*^{neo}*cKO*, 16974 ± 2719 versus 505 ± 128 cells; Figures 5-7A and 5-7B). The transcription factor Ascl1 is therefore required for RGLs in the adult DG to respond to neurogenic stimuli, and its loss renders RGLs inactive and unable to respond to their environment.

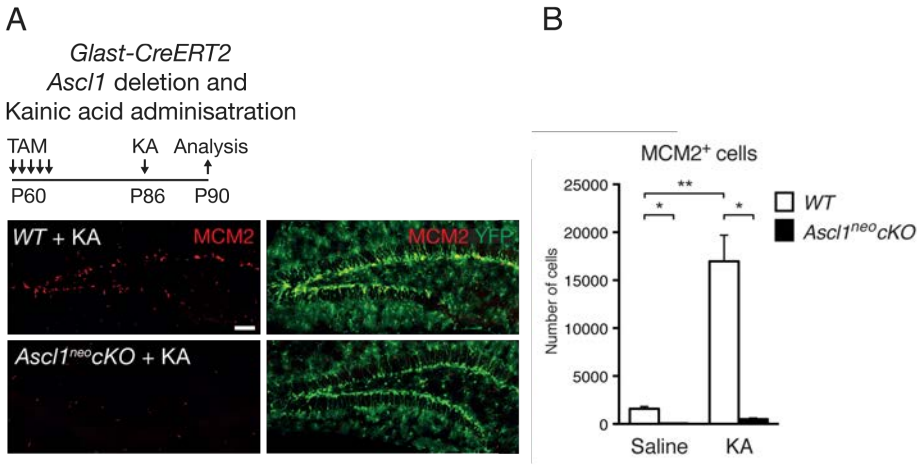

Figure 5-7 RGLs do not respond to neurogenic stimuli after *Ascl1* deletion (legend next page)

Figure 5-6 RGLs do not respond to neurogenic stimuli after *Ascl1* deletion

(A and B) Kainic acid (KA) administration to *WT* and *Ascl1^{neo}cKO* mice that had received TAM at P60 and analysis 4 days later to study the response of RGLs after *Ascl1* inactivation. Immunostaining for MCM2 and YFP show that KA greatly induces precursor activation in the DG of *WT* mice, but it has no effect on *Ascl1*-deficient cells. Quantification of the total number of MCM2^{+} cells in the DG of *WT* and *Ascl1^{neo}cKO* mice four days after either saline or KA injection illustrates the lack of a neurogenic response in *Ascl1^{neo}cKO* animals. n = 3 (B) for Saline in both genotypes, and KA in *WT*; 2 (B) for KA in *Ascl1^{neo}cKO*. * $p<0.05$, ** $p<0.01$. Scale bar = 40μm (A).

Chapter 6 Results

Targets of Ascl1 in stem cells of the adult dentate gyrus

Ascl1 is required for the response of stem cells to their environment: reception of a signal modulates Ascl1 expression to generate a response, which in this case will mean either to enter the cell cycle to divide or not. One important question that remains to be addressed is what mediates this role, i.e. what are the targets of *Ascl1* in adult hippocampal stem cells. To understand this we started by examining the genome-wide binding of *Ascl1* in hippocampal stem cells and established possible genes that could be performing this role. From here I was then able to select a number of genes to validate *in vivo*, that I will be describing next.

6.1 Genome-wide investigation of Ascl1-bound genes

In order to identify possible target genes mediating the proliferative role of *Ascl1* in hippocampal stem cells, we performed chromatin immunoprecipitation-sequencing (ChIP-seq). With this approach we were able to identify, in a genome-wide manner, Ascl1 binding sites in neural stem cells derived from the adult hippocampus (AH-NSCs). Next, using the Genomic Regions Enrichment of Annotations Tool (GREAT), we established a list of genes associated with an Ascl1 binding peak to be able to search for target candidates (see Table A1 1). Appendix 1 contains the information and data regarding the ChIP-seq analysis, which was performed by Ben Martynoga (see also Andersen et al., 2014).

Because we had an extensive list of genes bound by Ascl1, we decided to intersect these with differentially expressed genes in quiescent versus active adult-like stem cells of already published data sets. First, I intersected Ascl1-bound genes with genes that were downregulated during quiescence in Martynoga et al., 2013. Martynoga and colleagues characterised a cell culture model of NSC quiescence, where exposure of embryonic stem cell-derived NSC cultures to BMP4 leads to their acquisition of a quiescent state. Quiescence is a fundamental characteristic of stem cells in adult tissues. Since Ascl1 regulates the exit of these cells from their dormant state, we reasoned that those genes that were downregulated after BMP4 treatment in NSC cells would be some of the genes important during stem cell proliferation and, therefore, possible Ascl1 targets. Of the 4079 Ascl1-bound genes, 500 of them were common to the total 1957 genes down in quiescence NSCs (Figure 6-1A; see Table A1 2 in Appendix 1 for a list of overlapping genes). Among the genes that are both

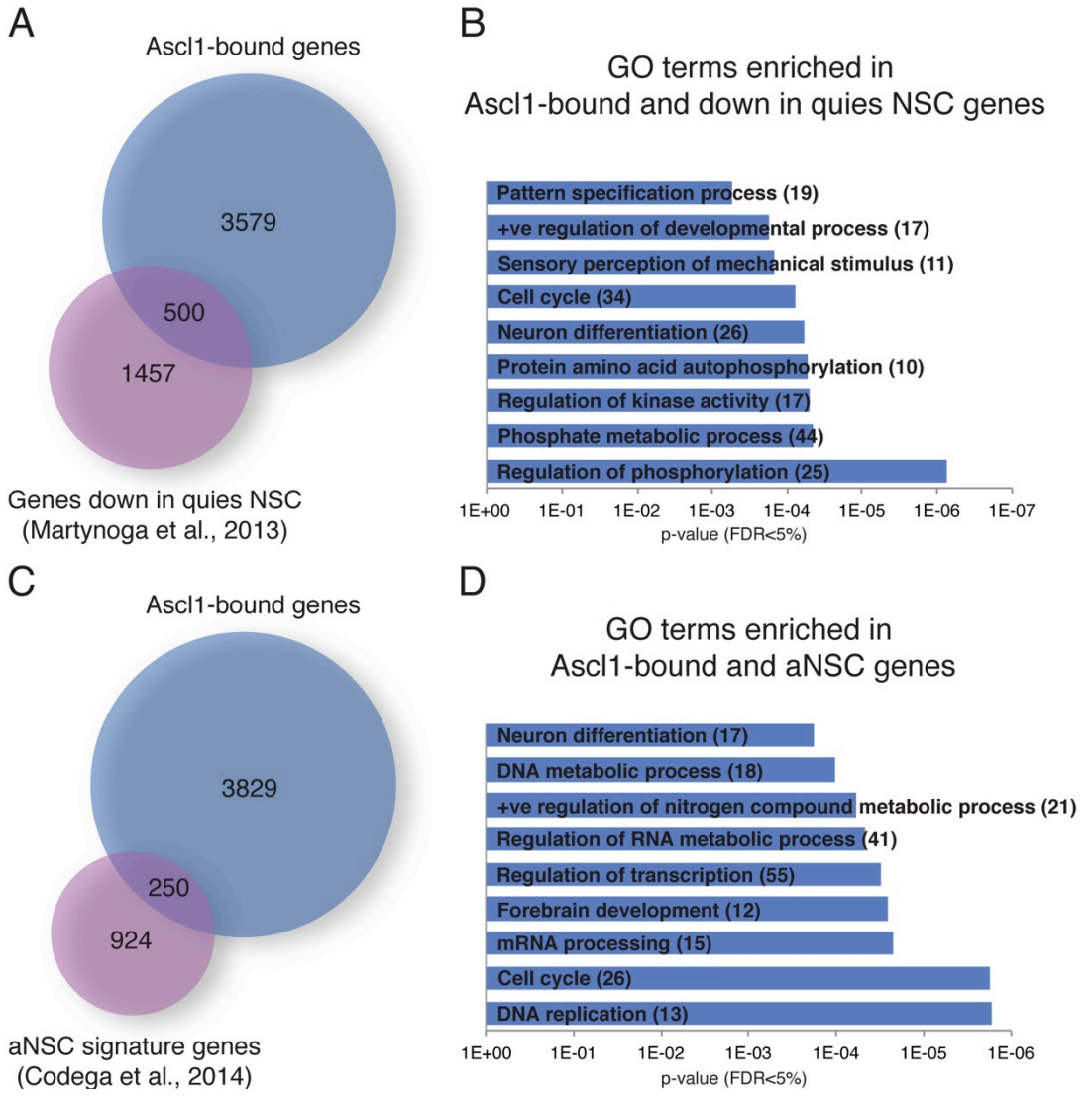

Figure 6-1 Investigation of Ascl1-bound genes in AH-NSCs

(A) Venn diagram showing the overlap (purple) between genes bound by Ascl1 in adult hippocampus-derived NSCs (AH-NSCs, blue) and genes that are downregulated in quiescent NSCs (quies NSC, pink) after BMP4 treatment from Martynoga et al., 2013. **(B)** Gene ontology (GO) analysis of the 500 genes common for both sets of genes from (A) reveals that the terms most enriched are those related to kinase activity and cell cycle regulation, among others. **(C)** Venn diagram showing the overlap (purple) of genes that were bound by Ascl1 in AH-NSCs (blue) and those that were enriched in activated NSCs (aNSCs, pink) from Codega et al., 2014. **(D)** GO analysis of the 250 genes common for both sets of genes from (C) reveals that the terms most enriched are those related to cell cycle processes. See also Appendix 1 for the full lists of genes in (B, D). (B and D) The X-axis values correspond to DAVID p-values. All terms reported have a false discovery rate (FDR) <5%. The total number of genes in each term is shown in brackets.

bound by Ascl1 and downregulated in quiescence there are known Ascl1 targets such as *Dll1* and *Dll3* (Castro et al., 2011). In addition, Gene Ontology (GO) analysis using DAVID showed these genes were mainly involved in the regulation of phosphorylation and kinase activity, as well as, importantly, the regulation of the cell

cycle (Figure 6-1B). Some of the cell cycle-related genes include *Ccnd1*, *Cdc6*, *Ccna1* and *E2f1* (see Table A1 3 in Appendix 1 for a full list of genes included in each term).

Next, I intersected the list of Ascl1-bound genes with a recently published list of SVZ genes enriched in activated NSCs (aNSCs) compared with quiescent NSCs (qNSCs; Codega et al., 2014). Fiona Doetsch's group isolated stem cells from the adult SVZ and used GFAP, prominin and the presence or absence of EGFR to distinguish active versus quiescent NSCs, respectively. From this analysis, I found 250 genes to be present in both lists (Figure 6-1C, and see Table A1 4 in Appendix 1 for a list of overlapping genes). GO analysis of these 250 common genes between Ascl1-bound and aNSCs from Codega et al., 2014 showed cell cycle-related processes to be the most represented terms (e.g. GO terms "DNA replication", "Cell cycle", "mRNA processing" and "DNA metabolic process", Figure 6-1D). Cell cycle regulators included in these terms are *Cdc6*, *Rrm2*, *E2f1*, *Skp2*, *Ccnd1* and *Ccnd2* (see Table A1 5 in Appendix 1 for a full list of genes included in each term).

Intersection of the three lists (Ascl1-bound, down in quies NSC and aNSC signature genes) resulted in 95 common genes and revealed that cell cycle processes are also the ones predominantly enriched (see Tables A1 6 and A1 7 in Appendix 1 for a full list of genes and GO terms). Our *in vivo* studies described in previous chapters demonstrated that Ascl1 is required for stem cell proliferation, and the meta-analysis described here does but highlight the central role of *Ascl1* during this process.

6.2 Validation of Ascl1 targets

Cultured cells, in contrast to cells *in vivo*, are exposed to high concentrations of mitogens, thus biasing transcriptional annotations towards cell cycle-related terms. To establish whether cell cycle-related genes were among the direct targets of Ascl1 in adult hippocampal stem cells, and validate the analysis described above, we used FACS sorting to isolate YFP^+ cells from the DG of *WT* and *Ascl1^{neo}cKO* mice. We administered TAM for 5 consecutive days to 2 month-old mice carrying the *Glast-CreERT2* deleter as before, and dissected and dissociated their DG ready for FACS

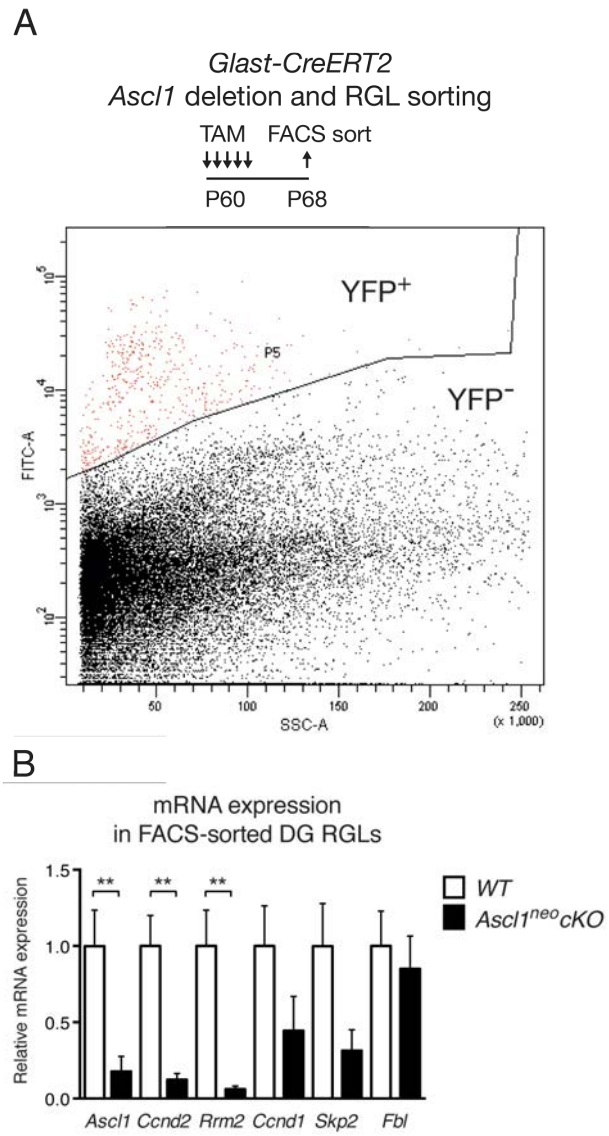

Figure 6-2 Validation of Ascl1 targets in the adult hippocampus

(A) Fluorescent-activated cell sorting (FACS) to isolate hippocampal RGLs was performed four days after the last of five TAM injections that started at P60. Representative FACS plot showing gating strategy. The X-axis represents the side scatter (SSC) and Y-axis represents the intensity of FITC (YFP expression). Red dots in P5 represent sorted cells (YFP^+). **(B)** Quantitative RT-PCR analysis of FACS-sorted YFP^+ RGLs from the hippocampus of *WT* and *Ascl1^{neo}cKO* mice shows that *Ascl1* expression, as well as expression of two Ascl1-bound cell cycle-related genes, *Ccnd2* and *Rrm2*, are strongly reduced in *Ascl1^{neo}cKO* cells. The expression of other Ascl1-bound cell cycle-related genes like *Ccnd1* and *Skp2* is not significantly reduced, and the expression of *Fbl* is unchanged. Expression levels are normalized to *Gapdh* and *Ppia* and are relative to *WT* expression. n = 4 (B) for *Rrm2* and *Fbl* for both genotypes; 6 (B) for *Ascl1*, *Ccnd2*, *Ccnd1*, *Skp2* for both genotypes. ** $p<0.01$.

purification 4 days after the last injection (Figure 6-2A). By then analysing gene expression by qPCR in FACS-sorted YFP^+ RGLs, we were able to determine if our

candidate genes were, as well as being bound by Ascl1, mis-regulated in *Ascl1^{neo}cKO*, and therefore possible direct targets.

Some of the genes tested were not detectable by qPCR (eg. *Cdc6*, *Ccna1* and *E2f1*, not shown), but of the ones detected, I found the cyclin *Ccnd2* and the ribonucleotide reductase M2 *Rrm2* to be significantly downregulated in *Ascl1*-deleted cells (Figure 6-2B). Expression of other cell cycle genes like *Ccnd1* and *Skp2* was reduced, albeit not significantly, and the expression of some non-cell cycle genes tested like *Fbl* was unchanged (Figure 6-2B). Together, this data demonstrates that *Ascl1* controls the proliferation of hippocampal stem cells by directly activating the expression of cell cycle genes.

6.3 Cyclins as Ascl1 targets

Both cyclins *Ccnd1* and *Ccnd2* are bound by *Ascl1*, and appeared to be both downregulated in quiescence in NSCs (*Ccnd1*), and enriched in aNSCs (both *Ccnd1* and *Ccnd2*). Interestingly, their importance in the self-renewing population of cells in the hippocampus has been previously established (Kowalczyk et al., 2004). I went on to further describe their expression in the RGLs of the DG. Ccnd2 is indeed expressed by radial progenitors in the DG (Figure 6-3, white arrow in top panel), and it is also expressed by non-radial IPCs (Figure 6-3, yellow arrowhead in top panel). Deletion of *Ascl1* by TAM administration at P60 showed that *Ccnd2* is completely lost from recombined cells one month later, as expected from the mRNA expression data.

Similarly, immunolabeling for Ccnd1 showed this protein to be highly expressed in RGLs in the SGZ (Figure 6-4A, white arrows in top panel). The proportion of Ccnd1^{+} RGLs was surprisingly higher when compared to any of the other markers of proliferation used so far (10-15% compared to less than 5% for MCM2, Figure 6-4B). A complete loss of Ccnd1 expression is, nevertheless, seen after *Ascl1* deletion (WT versus *Ascl1^{neo}cKO*, 11.2% ± 4.8% versus 0% Ccnd1^{+} RGLs; Figures 6-4A and 6-4B). This is different from what I observed after FACS-sorting analysis of *Ascl1^{neo}cKO* RGLs, where no significant difference in mRNA expression was observed between *WT* and *Ascl1*-deleted cells (see Figure 6-2B).

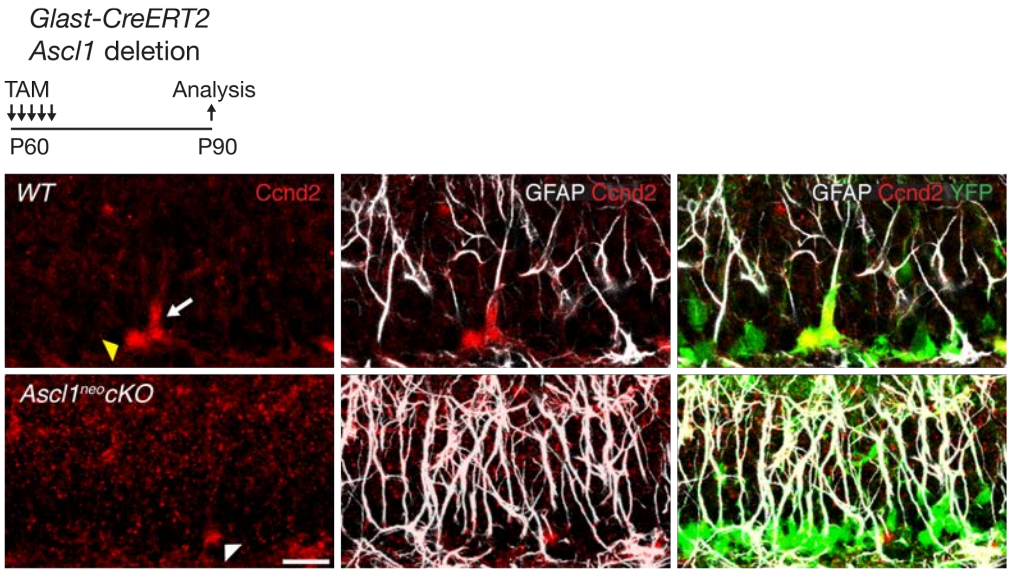

Figure 6-3 Ccnd2 expression in the adult DG

Labelling for Ccnd2, GFAP and YFP in the hippocampus of P90 *WT* and *Ascl1*^{neo}*cKO* mice that had received TAM at P60 shows that Ccnd2 is expressed in the SGZ of *WT*, but not *Ascl1*-deficient mice. In *WT* animals, Ccnd2 is expressed both by RGLs (white arrow, top panel) and IPCs (yellow arrowhead, top panel). In *Ascl1*^{neo}*cKO* mice, Ccnd2 expression is absent in YFP^{+} cells, indicating that Ccnd2 is a target of Ascl1 in the adult hippocampus. Note the presence of Ccnd2^{+} cells that are not part of the neurogenic lineage in the SGZ and therefore YFP^{-} (white arrowhead, bottom panel). Scale bar = 20µm.

Ccnd1 is bound by Ascl1 and its expression is lost in RGLs after *Ascl1* deletion, highlighting it as a likely direct target of this transcription factor. I previously showed Ascl1 to be expressed in RGLs early on, and before the activation marker MCM2, in response to the neurogenic stimulus KA. I hypothesised direct targets of Ascl1 to also be upregulated early on in response to neurogenic stimuli. To test this, I performed immunostaining for Ccnd1 24 hours after a single injection of KA in *WT* mice. This is the time when Ascl1 has already been induced, but MCM2 has not. Indeed, KA administration results in a dramatic induction of Ccnd1 just 24 hours after injection, following the pattern of induction seen for Ascl1 (Saline versus KA, 15.1% ± 2.2% versus 55.4% ± 4.1% Ccnd1^{+} RGLs; Figure 6-4C). These results show that Ascl1 controls stem cell activation by regulating expression of Ccnd1 and Ccnd2, among other cell cycle-related genes.

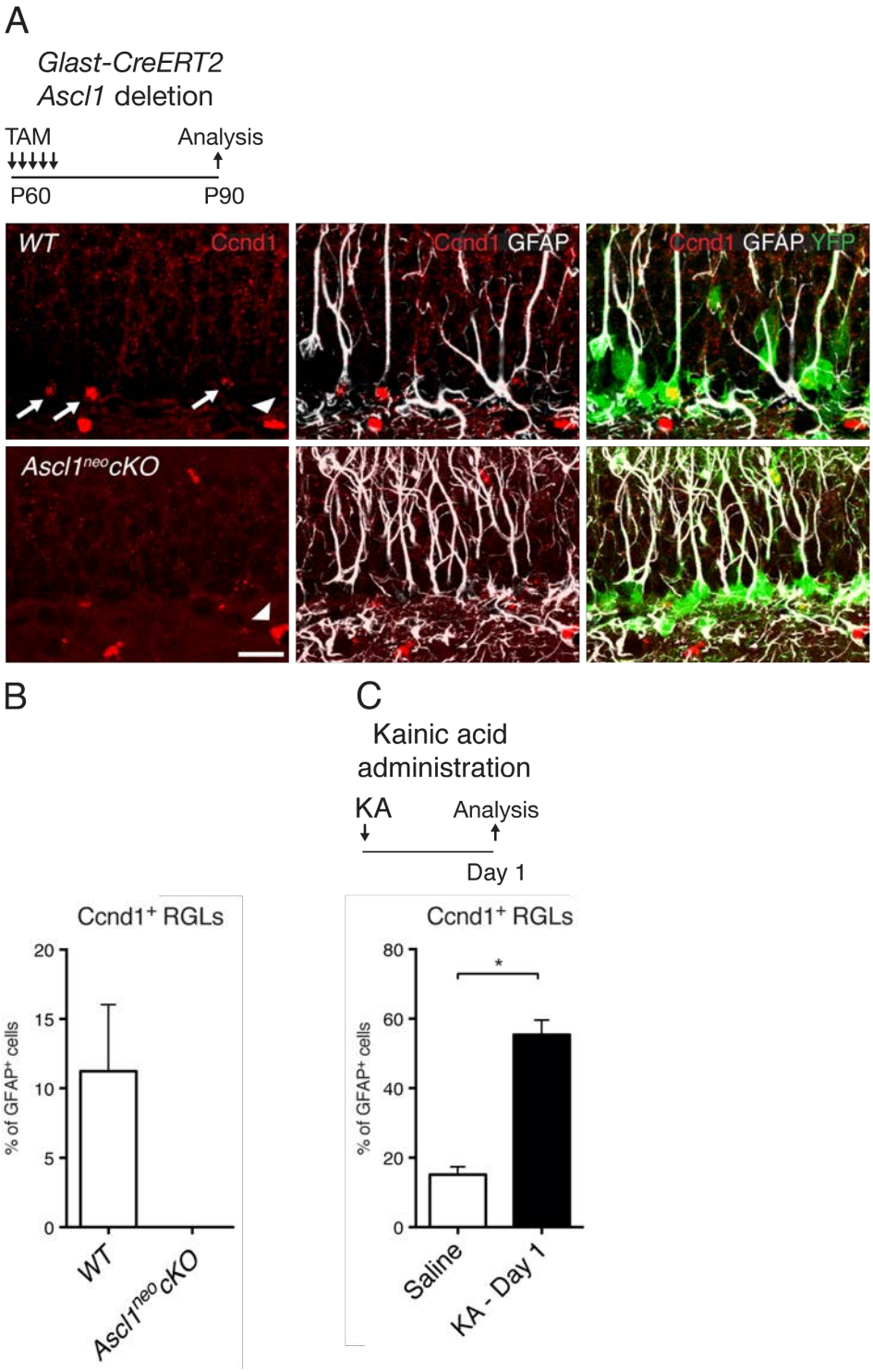

Figure 6-4 Ccnd1 expression in the adult DG (legend next page)

Figure 6-4 Ccnd1 expression in the adult DG

(A) Administration of TAM to *WT* and *Ascl1*^{neoflox} mice at P60 and immunohistochemical analysis one month later shows that Ccnd1 is expressed in a big proportion of *WT* RGLs (white arrows, top panel) but is absent in *Ascl1*^{neo}*cKO* cells, which have inactivated *Ascl1* (bottom panel). Note the presence of Ccnd1^{+} cells that are not part of the neurogenic lineage in the SGZ and therefore YFP^{-} (white arrowhead, top and bottom panels). **(B)** Quantification of the percentage of all GFAP^{+} radial cells that are Ccnd1^{+} shows that expression of this marker is completely absent in *Ascl1*-deficient stem cells. **(C)** Administration of kainic acid (KA) to *WT* animals and analysis of Ccnd1 expression in GFAP^{+} radial cells by immunohistochemistry revealed that the percentage of Ccnd1^{+} RGLs is drastically increased only one day after injection compared to saline-injected controls. n = 2 (C) for both treatments and (B) for *Ascl1*^{neo}*cKO*; 3 (B) for *WT*. * $p<0.05$. Scale bar = 20µm (A).

Chapter 7 Discussion

Although initially thought to be mainly confined to IPCs, Ascl1 expression is also observed in a third of the active radial GFAP-positive stem cells in the SGZ. This seemingly minor expression in steady physiological conditions is rapidly and significantly increased or decreased in response to neurogenic or anti-neurogenic stimuli, respectively. This dynamic pattern of expression together with results showing that loss of *Ascl1* completely blocks stem cell activation and subsequent neurogenesis suggest a model whereby signals from the surrounding environment converge on Ascl1 expression to modulate stem cell activity and consequently maintain tissue homeostasis under both physiological and pathological conditions.

In this chapter I will be discussing our results and putting them into context with what we know both within the hippocampal neurogenesis niche and within other adult stem cell niches. I have divided the body of the discussion into three main parts, which I believe correspond to the three fundamental aspects of this work. First I will be commenting on the signalling aspect of the work: the integration of external signals by stem cells and how this might converge onto *Ascl1*. Then I will discuss the molecular mechanism by which Ascl1 controls stem cell proliferation with a focus on cyclin proteins. Next, I aim to step back and consider the bigger picture, to explore the biological significance of this work and of Ascl1 function. I will end the chapter by postulating a few remaining questions and how they might be resolved and, finally, by drawing the general conclusions from the work presented.

7.1 Neurogenic and anti-neurogenic signals converge onto Ascl1 expression

7.1.1 Ascl1 as a switch for activation

Our results show that Ascl1 expression is rapidly modulated in hippocampal stem cells upon reception of environmental signals (Figure 7-1). This is a significant result, since it makes Ascl1 the first transcription factor found to integrate external stimuli and cell cycle regulation in the adult brain. What signals lie upstream of Ascl1 and how they control its regulation is, however, not yet known. A number of signalling pathways, nonetheless, are known to regulate stem cell activity and, the absolute phenotype we uncovered in *Ascl1* mutant mice, suggests that most of these known pathways act, ultimately, by regulating Ascl1 expression. By analogy with

other systems we might speculate how these signals interact with Ascl1 to regulate its activity (see Figure 7-2 for a schematic representation of how these signals might come together).

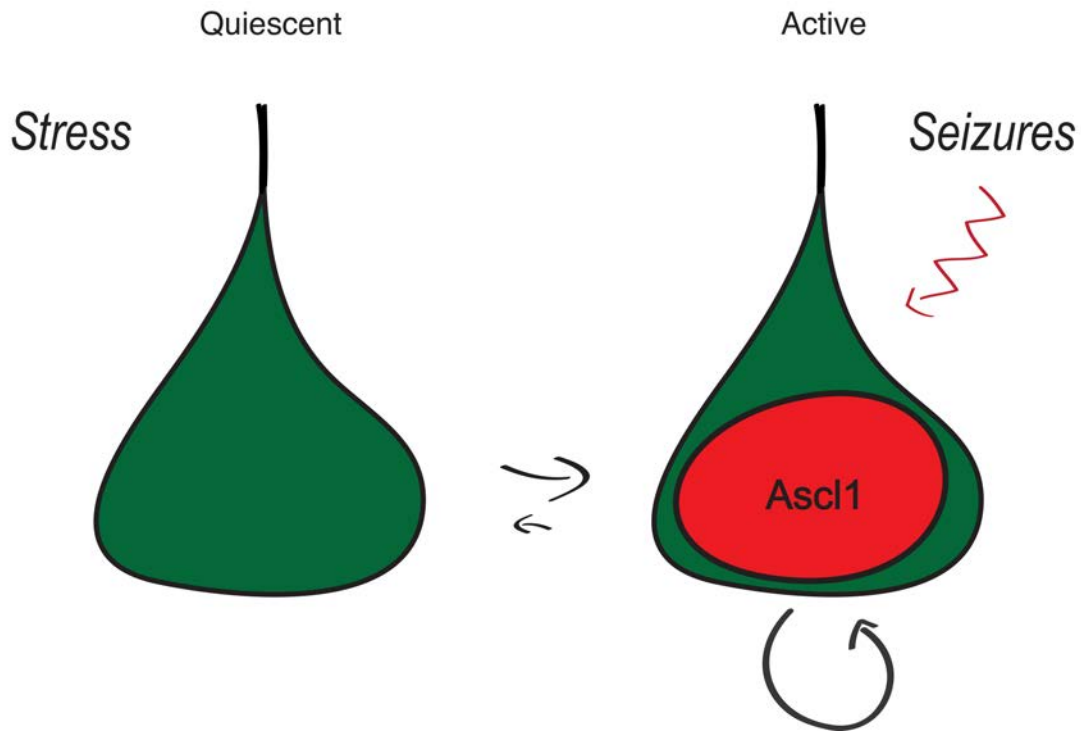

Figure 7-1 Ascl1 expression is regulated by extracellular stimuli

Ascl1 is rapidly modulated in hippocampal stem cells upon reception of neurogenic and anti-neurogenic stimuli to control the balance between quiescence and activation (see text for details).

Administration of a sub-seizure dose of KA resulted in a robust and consistent induction of Ascl1 expression in stem cells. KA is an agonist for the kainate type of glutamate receptors, and although it is widely used as a seizure-inducing agent and is known to activate stem cells in the adult brain, how it exerts its neurogenic function it is not clear. Presumably, KA, by over-exciting the hippocampal neuronal circuits is able to activate stem cells that were otherwise quiescent. A few years back, Karl Deisseroth and colleagues (Deisseroth et al., 2004), in view of the observation that a number of interventions affecting adult neurogenesis are also likely to modulate neuronal activity, went on to establish a possible link between excitatory neural activity and neurogenesis. By using adult rat hippocampal neural progenitor cell (NPC) cultures, they showed that excitation is

coupled to neurogenesis directly via Ca^{2+} channels and NMDA receptors present on the proliferative progenitor population. Moreover, they were able to show that excitation mediates neurogenesis by inhibiting the expression of the transcription factors Hes1 and Id2, and in turn by promoting the expression of NeuroD1. Both Hes1 and Id2 are negative regulators of Ascl1 (see below), but little difference in *Ascl1* expression was seen after excitation in this system. NPCs in culture are exposed to high concentrations of mitogens and are thus found to be actively dividing, making them more similar to IPCs *in vivo*. It remains to be determined whether quiescent RGLs *in vivo* respond to neuronal activity in a similar way that these neuronal progenitors *in vitro* do, and whether Ascl1 might be upregulated in such case.

An alternative hypothesis to explain the effect of KA on stem cells is the release of growth factors upon KA administration that in turn stimulate stem cell activation. VEGF mRNA, for example, has been shown to increase two hours after electroconvulsive seizures (ECS) in the rat hippocampus (Newton et al., 2003); and this VEGF induction was found to be necessary and sufficient for activation of quiescent stem cells of the rat DG (Segi-Nishida et al., 2008). Along these lines, deletion of VEGF in the adult mouse hippocampus using a *Nestin-CreERT2; VEGF$^{fl/fl}$* line resulted in a reduction of RGL proliferation (Kirby et al., 2014 Sfn poster presentation). In both cases VEGF, which could be acting both in a paracrine manner via endothelial cells, or in an autocrine manner as VEGF-C was shown to do in the SVZ (Calvo et al., 2011), appeared to be signalling through VEGFR-2 (Flk-1). Together, this evidence suggests an appealing model, where release of VEGF in response to KA acts on hippocampal stem cells to regulate Ascl1 expression via an as yet unknown intracellular signalling cascade.

Another well studied signalling pathway known to positively regulate neurogenesis in the adult brain and linked to neuronal activity is the Wnt/β-catenin pathway. Wnt ligands secreted by hippocampal astrocytes or, following an autocrine fashion, by neuronal progenitors themselves, are able to regulate progenitor proliferation and later differentiation (reviewed in Varela-Nallar and Inestrosa, 2013). Two recent publications suggest that both physiological and pathological stimuli, namely neuronal activity and ageing, modulate stem cell activity by

regulating Wnt signalling, through the expression of the two Wnt inhibitors sFRP3 and Dkk1, respectively (Jang et al., 2013, Seib et al., 2013). In crypt intestinal stem cells, Ascl2, a transcription factor paralog to Ascl1, is a master regulator of crypt stemness (van der Flier et al., 2009) and is under direct regulation of Wnt signalling (Schuijers et al., 2015). An intriguing possibility is that of Ascl1 being a target of Wnt in stem cells of the hippocampus. However, the expression pattern of the Wnt receptor Frizzled 1 (Fzd1) in the adult DG suggests that Wnt acts mainly on IPCs (Varela-Nallar et al., 2014 Sfn poster presentation). Moreover, cre-mediated deletion or short hairpin RNA (shRNA)-induced silencing of β-catenin in $Sox2^+$ cells, suggests that disruption of Wnt signalling in the DG blocks neuronal differentiation without affecting the $GFAP^+$ $Sox2^+$ stem/progenitor cell compartment (Kuwabara et al., 2009). Examination of Wnt-responsive cells in the SGZ of the adult DG will shed light on the role of this signalling pathway in the RGL population.

While the mechanism by which KA induces Ascl1 is still unclear, the mechanism by which loss of Notch signalling results in an increase of Ascl1 expression can be inferred from studies in the embryonic brain. Notch signalling induces the expression of Hes factors, and these directly repress *Ascl1* transcription (reviewed in Imayoshi and Kageyama, 2014). RBPJκ is part of the transcriptional activator complex responsible for *Hes* expression, and its deletion renders the Notch pathway non-functional, and hence leads to stem cells being rapidly activated, since Notch is required for stem cell maintenance (Ehm et al., 2010). This activation, prompted by the removal of an anti-neurogenic signal, is most likely brought about by the release of *Ascl1* inhibition. Deletion of *Ascl1* in *RBPJκ* mutant mice would establish whether the stem cell activation seen in the latter is, as expected, Ascl1-dependent. In our experiment, as it was the case for the KA experiment, we found Ascl1 to be induced before stem cells express MCM2 and exit quiescence, with an increase in the presence of Ascl1-only positive stem cells, supporting the idea that stem cell activation is Ascl1-dependent.

Anti-neurogenic signals can, not only target and inhibit *Ascl1* transcription, but they can also target Ascl1 protein and prevent it from executing its role. BMPs are important for the maintenance of quiescent RGLs (Martynoga et al., 2013, Mira et al., 2010), and by inducing expression of Id proteins, which can physically interact

with Ascl1 DNA-binding partners, the E-proteins, they repress Ascl1 activity (Jen et al., 1992, Bertrand et al., 2002). In the SVZ, Id1 expression characterises the quiescent B1 stem cells (Nam and Benezra, 2009), and deletion of all three Id genes results in an inability of stem cells to retain their stemness (Niola et al., 2012). Presumably, this phenotype could be linked to the loss of inhibition of Ascl1 activity.

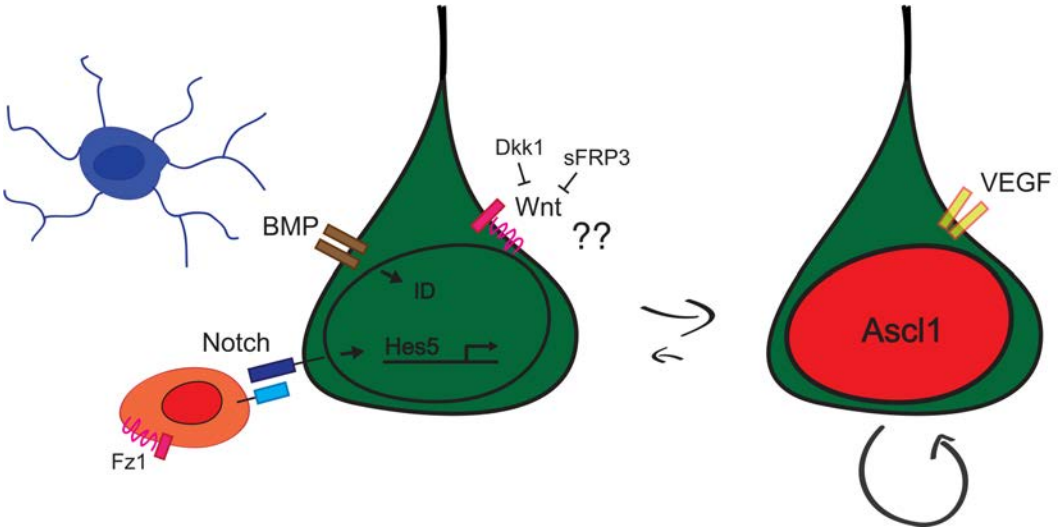

Figure 7-2 Extrinsic and intrinsic signals converge to regulate Ascl1 expression

Diagram illustrating the different signalling pathways known to regulate stem cell activity and how some of them might converge onto Ascl1 activity, both at the mRNA and protein levels (see text for details).

No doubt, more signals affecting stem cell activity will be identified in the near future. However, the knowledge we possess so far represents a good starting point for further studies (Figure 7-2). Moreover, it will be of interest to investigate whether strong pathological insults to the niche can induce the activation of an alternative mechanism of stem cell activity that is Ascl1-independent. Preliminary data from one of our collaborators, Juan Manuel Encinas, suggests that this might be the case. In a model of temporal lobe epilepsy (MTLE), intrahippocampal injection of KA, a procedure that delivers high concentrations of KA directly to the DG, results in a very significant activation of quiescent RGLs in *WT* mice three days after injection. This activation, in accordance with an activation-dependent deforestation hypothesis (Encinas and Sierra, 2012), results in an accelerated depletion of the stem cell pool. In *Ascl1^{neo}cKO* mice, there was a significant reduction in the percentage of dividing RGLs after KA injection in the SGZ, a result that would lead to a partial

preservation of the RGL population. However, in contrast to what we observed after an i.p. injection of KA at a concentration that does not induce observable seizures, our collaborator found a considerable proportion of stem cells that were able to proliferate ($BrdU^+$ radial NSCs: 45% in *WT* mice versus 15% in *Ascl1^{neo}cKO* mice; Encinas J.M., personal communication). This might suggest that an alternative mechanism for stem cell activation exists but it only comes into play after strong insults to the brain.

Once inside the cell, a few transcription factors are known to be involved in mediating the response to dormancy or activation signals. One of the transcription factors thought to mediate the activity of extracellular signals is FoxO3, which is inactivated in response to the insulin/IGF-1 signalling pathway. FoxO3 acts in the NSC pool to induce a transcriptional programme that promotes quiescence (Renault et al., 2009). Genome-wide analysis of FoxO3 and Ascl1 targets in cultures of adult neural progenitor cells revealed that these two transcription factors share common targets, and that binding of FoxO3 to these targets in quiescent cells antagonises Ascl1's activity (Webb et al., 2013), suggesting a mechanism by which a balance of quiescence versus activation transcription factors might regulate NSC homeostasis.

In contrast, and as an example of another factor, as Ascl1, responsible for positively regulating stem cell function, we know of the orphan nuclear receptor TLX, which has been shown to promote proliferation of RGLs of the adult hippocampus. This action was shown to be mediated extrinsically by Wnt7a in an autocrine/paracrine fashion, and intrinsically by p21 in a p53-dependent manner (Niu et al., 2011, Qu et al., 2010). How and if TLX is regulated by neurogenic stimuli is, however, not yet known. Interestingly, though, TLX was shown to activate *Ascl1* and induce neuronal lineage commitment in cultured adult rat hippocampus-derived progenitors (Elmi et al., 2010). Whether TLX is acting upstream of *Ascl1 in vivo* to promote stem cell proliferation will remain an important question to address in the future.

7.1.2 Fine-tune of the neurogenic output by Ascl1 regulation

Considering our results together with the above analysis, a model emerges where Ascl1 expression is controlled both at the mRNA and protein levels to modulate the rate of hippocampal neurogenesis. Our results obtained with mice carrying the hypomorphic allele *Ascl1*^{neoflox}, as well as those carrying a copy of the *Ascl1* null allele (*Ascl1 Δ*) support such a model. Reducing levels of *Ascl1* transcripts is translated into a reduction of the fraction of RGLs that divide in the adult DG, and the extent of the reduction appears to be strongly correlated with *Ascl1* expression levels. Moreover, decreasing the proliferating portion of RGLs is directly correlated with the neurogenic output, as shown by the considerable loss of DCX^{+} immature neurons in *Ascl1*^{neoflox} mice.

The above model is also supported by the phenotype observed in RGLs in animals where Ascl1 is regulated at the protein level. A current member of our laboratory has created a line where the E3 ubiquitin-protein ligase *Huwe1* is conditionally deleted in RGLs in adult mice. Huwe1 interacts with Ascl1 protein to target it for proteasomal degradation and, therefore, control its stability. When *Huwe1* is deleted in hippocampal stem cells, we observe an increase in Ascl1 positive RGLs and, consequently, an increase in the fraction of active stem cells (Urbán N., manuscript in preparation). In the embryonic brain, differential levels of Ascl1 are also involved in mediating the response to extracellular signals. Ascl1 protein stability is regulated by the PDK1/Akt signalling pathway, which, in itself is being regulated by the extrinsic growth factor IGF-1, to control the production of neocortical GABAergic neurons (Oishi et al., 2009). Altogether, these results strongly support the idea that tight regulation of the levels of Ascl1 is important for maintaining an adequate balance of quiescence and activation in stem cells of the adult hippocampus and for ensuring that the adequate number of neurons is generated.

7.2 Ascl1 has a crucial role in hippocampal stem cell proliferation

7.2.1 Ascl1 acts in a cell-autonomous manner in hippocampal stem cells

The results obtained with the mouse models of *Ascl1* deletion point to a central role of this proneural protein during the cell cycle exit and proliferation of adult hippocampal stem cells. But because we have shown Ascl1 to be expressed in both early progenitors and activated type 1 cells, it was important to elucidate where Ascl1 was acting. Ascl1 could be cell autonomously controlling stem cell proliferation, leading to the rest of the phenotype in the lineage, or Ascl1 could be controlling RGL activity in a non-cell autonomous way as a secondary consequence of the loss of IPCs.

Two possible scenarios can be envisaged for the latter idea of IPCs being the primary cause and loss of active RGLs being a secondary consequence in the phenotype we observe. In the first scenario, IPCs could, in normal conditions, be exerting a positive effect on RGLs by, for example, secreting growth factors that affect their activity. Loss of this positive feedback, through loss of IPCs, could lead to a block of RGL proliferation. A recent publication reported Ascl1 to be critical for the maintenance and tumorigenicity of glioblastoma cancer stem cells (GBM CSCs), and highlighted a possible mechanism by which Ascl1 could control proliferation in a non-cell autonomous way. By binding to and repressing the Wnt negative regulator *Dkk1*, Ascl1 activates Wnt signalling, which in turn promotes proliferation (Rheinbay et al., 2013). In the zebrafish injured retina, induction of *Ascl1a* and inhibition of Dkk1-mediated Wnt repression is also a mechanism utilised for successful regeneration (Ramachandran et al., 2011).

We examined the possibility of Ascl1 controlling hippocampal stem cell proliferation by modulation of Wnt signalling by interrogating the genes bound by Ascl1 in AH-NSCs. Only a small fraction of these genes overlapped with those genes bound by Ascl1 in GBM CSCs. Well-characterised Ascl1 targets like *Dll1*, for example, were common sites in the two cell types. However, many more sites were found only in AH-NSCs, including those of cell cycle regulators like *E2f1*, *Ccnd2*, *Ccna1* and *Skp2*. And importantly, we found no Ascl1 binding site near the Wnt

regulator gene *Dkk1* in AH-NSCs (see Figure A1 2 in Appendix 1). This suggests that Ascl1 does not control stem cell proliferation in the hippocampus by regulating Wnt signalling.

We next went further and investigated the possibility of an as yet unknown positive feedback signal from IPCs to RGLs being disrupted and therefore affecting stem cell activity with two different analyses: deletion at an early time-point and mosaic deletion. In both cases the progeny of *WT* cells was present and could thus still provide any required stimulation. In no case we found *Ascl1* mutant cells being rescued by the neighbouring progeny and dividing. The possibility of the hypothetical positive feedback signal being below a certain necessary threshold is unlikely since we found the absence of dividing RGLs to be independent of the recombination efficiency in the mosaic analysis (see Figure 3-12D).

In the second scenario of a non-cell autonomous effect of Ascl1, IPCs could be part of a negative feedback loop to maintain RGLs in a quiescent state. A number of recent reports have provided evidence that IPCs, by activating Notch signalling through Notch ligands expressed on their surface promote stem cell quiescence (Hodge et al., 2012, Lavado et al., 2010, Kawaguchi et al., 2013, Lavado and Oliver, 2014). In this case, and because the loss of IPCs will result in an activation of RGLs, we would expect to find dividing RGLs in situations where IPCs are totally absent. Our analysis at P90, one month after *Ascl1* deletion, shows that the progeny is already completely absent but no proliferation is taking place. When analysing the hippocampus of *Ascl1*^{neo}*cKO* mice five months after deletion, we still find RGLs to be in a quiescent-like state, with no proliferation and no exhaustion of the stem cell population, which has been reported for those mutant mice lines where stem cell maintenance is disrupted (Mira et al., 2010, Ehm et al., 2010, Andreu et al., 2015, Ables et al., 2010). What's more, at no time point do we observe formation of new neurons or an increase of GFP-expressing cells, which we would expect if there was a rise in proliferation immediately after TAM administration. All together, these results show that Ascl1 controls stem cell proliferation in a cell-autonomous manner.

One separate and important aspect to discuss concerning the function of Ascl1 in stem cell proliferation is the idea that the cre recombinase could be having

an effect on proliferation on its own, as suggested by the experiments with *Ascl1*^{flox} animals. Total proliferation was significantly reduced in *Ascl1 WT* mice compared to *Ascl1*^{flox} mice. Similarly, proliferation in RGLs in these mice was highly, but not significantly reduced (see Figures 3-9 and 3-10). Since the animals analysed were littermates, the only difference between them was the presence (*WT*) or absence (*Ascl1*^{flox}) of the *GlastCre-ERT2* deleter, suggesting that indeed cre is the responsible for the differences observed. Despite the reduction of proliferation having been already described in mammalian cells in the presence of cre recombinase (Loonstra et al., 2001), this was an unexpected result for us. Our main phenotype is one affecting proliferation, and it is therefore important to understand the implications of this idea.

Ascl1^{neo}*cKO* animals carry the cre recombinase, and it might be argued that the difference in proliferation observed between these animals and their *Ascl1*^{neoflox} cre-less counterparts is in fact a consequence of this presence rather than the loss of *Ascl1* in *Ascl1*^{neo}*cKO* animals. There are a number of arguments against this idea. Firstly, we know that loss of Ascl1 significantly affects proliferation in a cre-independent manner because disruption of *Ascl1* transcription by the presence of the *PGK promoter-neo* cassette results in a strong block in proliferation in *Ascl1*^{neoflox} animals that is restored when the cassette in the *Ascl1* locus is removed. Similarly, both total and RGL proliferation are significantly different between Ascl1 *WT* and *Ascl1cKO* animals, both of which carry the cre recombinase. Finally, and following a similar trend of thought, when both *Ascl1 WT* and *Ascl1*^{neo}*cKO* animals receive KA injections, only *Ascl1 WT* stem cells are able to respond, while cells that have deleted *Ascl1* remain unresponsive to the stimulus. All together, our results, even though they point to a cre-dependent proliferation effect in the DG, they show that Ascl1 is able to regulate proliferation independently of any cre effect.

7.2.2 Ascl1 regulates cyclin D genes in hippocampal stem cells

Our gene expression analysis in FACS-isolated *WT* and *Ascl1* mutant cells, together with protein expression analysis, indicate that Ascl1 promotes the cell cycle progression of RGLs by directly regulating, among other cell cycle-related genes, the cyclins *Ccnd1* and *Ccnd2* (Figure 7-3). D-type cyclins are essential proteins for the

progression through the G_1 phase of the cell cycle. In early G_1, D cyclins activate cyclin-dependent kinases 4 and/or 6 (CDK4/6) to initiate the phosphorylation of the retinoblastoma (Rb) family of proteins (Rb, p107, p130). Rb is a central component of a transcriptional repressor complex that, by inhibiting the binding of E2F transcription factors to their target genes, inhibits the promotion of the cell cycle. Phosphorylation of Rb leads to the release of E2Fs and the activation of the genes required for G_1 progression (reviewed in Coqueret, 2002, Satyanarayana and Kaldis, 2009).

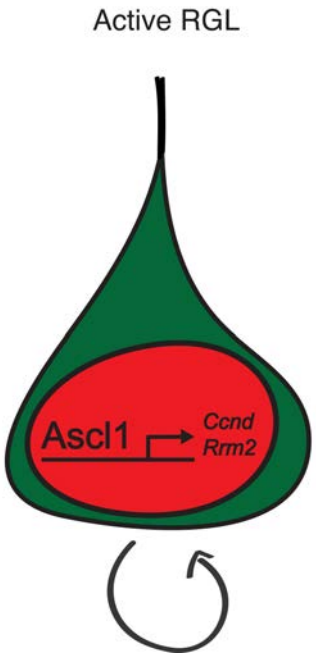

Figure 7-3 Ascl1 directly regulates cell cycle genes to control hippocampal stem cell proliferation

Ascl1 binds to and activates *cyclin D* genes as well as the *ribonucleoside reductase M2* (*Rrm2*) gene to control hippocampal stem cell activity (see text for details).

While CDK levels remain constant through the cell cycle, expression of cyclins varies throughout. In particular, the expression of D-type cyclins is largely dependent on extracellular signals. Stimulation by mitogens is coupled with rapid cyclin D transcriptional induction, making these proteins a fundamental link between extracellular stimuli and cell cycle progression (Coqueret, 2002). We found both Ascl1 and Ccnd1 to be upregulated early upon induction of neurogenic stimuli, and

both do so before the induction of proliferation. Our results, therefore, are consistent with the mitogen-sensing role of cyclins.

The activity of CDK/cyclin complexes is also regulated by cyclin-dependent kinase inhibitors (CKIs), which are responsible for inhibiting CDK/cyclin complexes and induce cell cycle arrest. Two families of CKIs exist: the INK4 and the Cip/Kip families (Blomen and Boonstra, 2007). During quiescence, one of the Cip/Kip family members, *p27^{Kip}*, appears to be highly expressed and its levels decrease as cells progress through G_1. p27^{Kip} inhibits the formation of CDK2/cyclin E complexes, which are required for further Rb phosphorylation and progression through the G_1 restriction point (R) into S phase. Activation of CDK4/6-cyclin D complexes by mitogen stimulation can result in the sequestering of p27^{Kip} to release CDK2-cyclin E inhibition and promote proliferation (Blomen and Boonstra, 2007). Ccnd2 has been proposed to play a central role in the down-regulation of p27^{Kip} at the G_0-G_1 transition in a number of cell types by directly translocating p27^{Kip} from the nucleus to the cytoplasm for its degradation (Susaki et al., 2007). Very interestingly, a recent report has shown *p27^{Kip}* to be an important regulator of stem cell quiescence in the adult DG (Andreu et al., 2015). These observations, together with our results, point to the intriguing idea of the *Ascl1 - cyclin D - p27^{Kip}* axis as a possible mechanism for the induction of cell division in quiescent stem cells of the adult hippocampus.

Another member of the Cip/Kip family of CKIs, *p57^{Kip2}*, has been shown to play a role in the control of DG stem cell quiescence. p57^{Kip2} inhibits the formation of CDK2/cyclin E complexes, as its partner p27^{Kip} does. Quiescent RGLs in the DG, but not IPCs, express p57^{Kip2}, and conditional deletion of this CKI using a Nestin-driven *CreERT2* line, resulted in a transient expansion of the radial NSC pool due to the activation of the quiescent subpopulation (Furutachi et al., 2013). In the hematopoietic system, deletion of p57^{Kip2} leads to a compensatory upregulation of p27^{Kip} (Matsumoto et al., 2011), suggesting that these two factors may have overlapping roles. How they interact in the hippocampus to regulate RGL quiescence remains unknown.

Interestingly, mice mutant for *Ccnd2*, but not *Ccnd1*, showed severe defects in adult neurogenesis in the DG (Kowalczyk et al., 2004). While D-type cyclins are

in general able to compensate for the loss of one of their siblings, slight differences in the timing of expression in certain cell types can render them unable to perform functional compensation (Satyanarayana and Kaldis, 2009). This idea is illustrated by an elegant experiment where *Ccnd2*, when expressed in place of *Ccnd1*, is able to drive nearly normal development, suggesting that it is mostly the tissue-specific pattern of expression that drives the differences between these two cyclins (Carthon et al., 2005). In the case of the adult hippocampus, *Ccnd2* appears to be able to compensate for *Ccnd1* upon *Ccnd1* loss, but the opposite appears to not be the case. Another example where *Ccnd1* is unable to compensate for *Ccnd2* function is in ovarian granulosa cells, which show defective proliferation resulting in sterility in *Ccnd2* null mice (Sicinski et al., 1996). In the DG, this might be due to Ccnd1 being more broadly expressed than Ccnd2.

One disconcerting result that we observed is the discrepancy between *Ccnd1* mRNA expression in sorted stem cells and protein expression in the DG *in situ*. While Ccnd1 protein was absent when examined by immunohistochemistry, there was no significant down-regulation of *Ccnd1* transcript numbers by qPCR. *Ccnd1* levels can be regulated transcriptionally and post-transcriptionally by many different factors (Musgrove, 2006). One possibility is that the discrepancy we observe is due to differential regulation of the *Ccnd1* gene in different cell types. We performed RGL isolation by FACS three days after the last TAM injection in order to avoid collecting any YFP^+ progeny in *WT* samples; however, it is likely that some IPCs were sorted together with RGLs. If this was the case, we can envisage a scenario where *Ccnd1* expression in IPCs, regulated by other signalling cascades, is acting in an *Ascl1*-independent manner to induce neurogenesis, and that this is what we detect by qPCR. A number of signalling cascades are known to regulate *Ccnd1* expression, with Wnt/β-catenin being one of the better studied ones (Clevers, 2006). Wnt signalling plays an important role in both the proliferation and differentiation of IPCs in the adult DG (Varela-Nallar and Inestrosa, 2013).

Deletion of *Ascl1* in hippocampal stem cells also led to a significant down-regulation of *ribonucleoside reductase M2* (*Rrm2*) expression (Figure 7-3). *Rrm2* is regulated in a cell cycle-dependent fashion, and has been shown to inhibit Wnt signalling in HEK293 cells (Tang et al., 2007). It can be speculated, that if also true

for hippocampal stem cells, inhibition of the Wnt signalling pathway could be an important negative feedback mechanism to prevent stem cell over-proliferation. No studies have examined *Rrm2* expression or function in the adult DG.

It is very likely that Ascl1 regulates other cell cycle-related genes that we did not identify here. Our ChIP-seq analysis shows that Ascl1 binds to a significant number of genes involved in the cell cycle. However, we were not able to validate many of these due to limited starting material. Further expression analysis in *WT* and *Ascl1*-deficient RGLs, as well as studies with more mutant mouse lines, will shed light on the regulation of cell cycle progression in adult DG stem cells. One study, for example, has reported impaired hippocampal neurogenesis in mice lacking the transcription factor *E2f1*, a target of Ascl1 in AH-NSCs (Cooper-Kuhn et al., 2002). Whether *E2f1* is under the control of Ascl1 regulation in RGLs *in vivo* we do not know.

7.2.3 Diverse roles of Ascl1 during embryonic and adult neurogenesis

Neurogenesis in the embryonic brain is regulated primarily by proneural transcription factors, including Ascl1 and Neurog2. These factors play key roles in promoting neural progenitor cell cycle exit and neuronal differentiation (Bertrand et al., 2002). However, a more recent genome-wide characterisation analysis of the transcriptional targets of Ascl1 in the embryonic brain and in NSC cultures from our laboratory unravelled a novel function for this proneural gene. Ascl1 was shown to control the expression of a large number of positive cell cycle regulators, and to be required for normal progenitor divisions (Castro et al., 2011). The work described here illustrates another instance where Ascl1 is important for cell cycle progression. The comparison, nevertheless, brings about a number of topics worth discussing.

Of note is the differential proliferation dynamics in stem cells of the embryonic and adult brains. NPCs in the embryo are responsible for generating the diverse types of neurons and glia that build the entirety of the nervous system, and therefore need to continuously and rapidly proliferate. Stem cells in the adult hippocampus, on the other hand, are found primarily in a quiescent state, with only a small fraction of them dividing at one time (Urban and Guillemot, 2014). Therefore,

while we talk about proliferation in NPCs of the embryo, it would appear more accurate to refer to activation in hippocampal RGLs, since stem cells here need to be "awaken" before they proceed to proliferation. Here we show that, upon reception of extrinsic stimuli, quiescent RGLs induce Ascl1 and MCM2 expression. MCM2 is part of a pre-replication complex that marks not only cycling cells, but also activated cells that have not yet re-entered the cell cycle (Torres-Rendon et al., 2009, Stoeber et al., 2001). Deletion of *Ascl1* results in a loss of MCM2 expression, which suggests that RGLs are unable to become activated and therefore remain in a quiescent state. Whether activation and proliferation are coupled processes and are both regulated by Ascl1 in these cells it is not possible to determine, since we see a defect in the first one of them. Considering the phenotype observed in the embryonic brain after *Ascl1* loss, as well as the evidence presented showing that Ascl1 regulates cell cycle genes, we may assume it to be likely for Ascl1 to control both processes.

Similarly, having lost RGL proliferation and therefore IPC generation, it is not possible to determine what role Ascl1 plays in these cells. Because of their rapid proliferation dynamics, these cells resemble more embryonic NPCs, and therefore it may also be speculated that Ascl1 is important here. The use of an IPC-specific cre line would help establish whether this is the case. Ascl1 colocalises with Tbr2 in late IPCs, thereby the use of a *Tbr2-CreERT2* line crossed to *Ascl1cKO* mice, though not optimal, might be useful for this purpose.

During development, Ascl1 in NPCs is required for the process of neurogenesis in the ventral telencephalon (Casarosa et al., 1999). In the adult hippocampus, Ascl1 expression is downregulated before the onset of neuronal differentiation, and we see no colocalisation between this factor and markers of immature neurons like DCX. It is therefore unlikely that *Ascl1*, in the adult DG, plays a direct role in activating the programme of neurogenesis. In contrast, data obtained with *Huwe1* conditional mutant mice by another member of the laboratory show that Ascl1 needs to be downregulated in IPCs before the neurogenic programme can progress. Stabilization of Ascl1 protein by deletion of *Huwe1* resulted in a significant increase in the number of dividing RGLs in the DG, which is consistent with the idea of Ascl1 promoting the progression of the cell cycle. More interestingly, stabilization of Ascl1 protein also resulted in a block of neurogenesis,

indicating that Ascl1 protein needs to be downregulated for neurogenesis to proceed normally (Urbán N., manuscript in preparation). Therefore, Ascl1 might be playing other roles in the adult DG that we are not able to observe with our mouse model.

Next, and related to Ascl1 levels, we consider the relevance of the dynamic expression of Ascl1. The multipotent state of embryonic NPCs has been shown to correlate with the oscillatory expression of a number of fate determination factors, including Hes1, Ascl1 and Olig2. On the other hand, accumulation of Ascl1 during G_1 phase is a decisive sign for neuronal determination (Imayoshi et al., 2013). Whether oscillatory behaviour of proneural genes is also characteristic of adult stem cells it now under investigation (Kageyama R., personal communication). Our work shows that deletion of *Ascl1* leads to a complete block of proliferation, but Ascl1 protein expression is only observed in a third of active RGLs. Similarly, Ccnd1 is expressed in a much larger proportion of RGLs than those expressing Ascl1. These patterns could be an indication that Ascl1 expression also oscillates in adult hippocampal stem cells.

Given the crucial role of Ascl1 during embryogenesis, it was important to evaluate the possibility that the phenotype we observed in adult *Ascl1^{neo}cKO* animals was not brought about by developmental defects. The first argument against this idea is that DG of adult *Ascl1^{neo}cKO* animals looks normal. If *Ascl1* were required during development for hippocampal granule cell generation, we would expect a smaller and undeveloped DG to be formed, as it is the case for the DG in animals that have lost the bHLH factor *Neurog2* (Galichet et al., 2008). This initial piece of evidence suggests that Ascl1 function might be dispensable during early hippocampal development. Moreover, we not only observe the same phenotype in our *Ascl1cKO* animals, but we were also able to show that there is a switch in the requirement of *Ascl1* from early postnatal to adult stages. When *Ascl1* was deleted at P7, RGLs continued to proliferate three days later (Andersen et al., 2014). These results nicely fit with observations made in *Ccnd2* mutant mice, where DG cell proliferation appears to be dependent on *Ccnd2* only after the first few weeks of life (Ansorg et al., 2012). Ccnd1 appears to be able to partially compensate for *Ccnd2* loss for at least the first two weeks of life, when the reduction in proliferation in the SGZ is only about 60%. After this time, between P14 and P28, however, there appears to be

a switch with Ccnd2 becoming an essential requirement for on-going neurogenesis. Therefore, it appears likely that a switch in the control of RGL proliferation takes place during early postnatal stages, with the activation of a mitogenic pathway involving *Ascl1* and *Ccnd2*: from embryonic *control* to adult mitogenic *regulation* (see section 1.3.4 for definitions of control and regulation).

7.2.4 Transcriptional regulation by Ascl1

Our results show that Ascl1 controls stem cell activation in the adult hippocampus by regulating expression of cell cycle-related genes. The mechanism by which Ascl1 regulates transcription is, however, not yet completely understood. Gene expression is regulated through the combined activity of many regulatory elements, including promoters, which are normally found near the transcriptional start sites (TSS) of genes, and elements that are located at a greater distance from the TSS, such as enhancers.

Enhancers are non-coding sequences that activate, and can greatly increase, promoter-mediated transcription of their target genes. Enhancers are similar to proximal promoter regions in that they are organized as a series of cis-acting elements that are bound by trans-acting regulatory proteins. They can be distinguished from promoter elements, however, by their ability to act at a distance and to do so either upstream or downstream from the promoter they control. Upon binding of specific transcription factors, recruitment of transcriptional coactivators and chromatin remodelling proteins occur on the enhancers. Here, the protein complexes are thought to facilitate DNA looping, bringing enhancer protein complexes into close physical proximity to those on the promoter, and allowing promoter-mediated gene activation.

Enhancers and their transcription factors have, therefore, prominent roles in the initiation of transcriptional regulation of gene expression. Importantly, combinatorial transcription factor occupancy can lead to diverse types of transcriptional output, resulting in discrete and precise patterns of transcriptional activity at different developmental stages or different cell types (Spitz and Furlong, 2012).

The generation of genome-wide maps of specific chromatin marks has allowed the successful identification of enhancers and other regulatory elements in the genome. The use of ChIP-seq techniques, for example, has provided a tool for the identification of genome-wide binding profiles of both DNA-binding proteins and chromatin remodelling complexes that are enriched at specific regulatory elements. For instance, histone 3 lysine 4 monomethylation (H3Kme1) was found to mark enhancers, and distinguish them from promoters, which are marked by trimethylation at this site (H3K4me3). Similarly, histone 3 lysine 27 acetylation (H3K27ac) has been associated with enhancers, and has been shown to discriminate active from poised enhancers, which contain H3K4me1 alone. Another element that has been used for enhancer identification is the acetyltransferase and transcriptional coactivator p300, which is a protein associated to enhancers. Therefore, a combination of histone mark identity and presence of p300 have been previously used to identify and predict the activity of transcriptional enhancers throughout the genome (Rada-Iglesias et al., 2011, Heintzman et al., 2009, Creyghton et al., 2010).

In our work we have used ChIP-seq to identify Ascl1-binding sites in the genome of AH-NSCs. Analysis of these binding events showed that most Ascl1-binding sites are located between 5kb and 500kb away from a TSS (see Figure S7 in Andersen et al., 2014), suggesting that Ascl1 binds mainly distal enhancers in AH-NSCs. Indeed, we show that a large fraction of Ascl1-binding sites overlap enhancers present in proliferating NSCs (from Martynoga et al., 2013), while almost half of enhancers present in proliferating NSCs are bound by Ascl1. In agreement with this data, Raposo et al. (2015) showed that Ascl1 binds predominantly distal enhancer regions in differentiating NSCs.

The study by Raposo and colleagues (2015) also shed light on the possible mechanism of action of Ascl1 activity. They showed that Ascl1 acts as a pioneer transcription factor when regulating gene expression in its native context. In a previous study, Wapinski et al. (2013) had uncovered that, during fibroblast reprogramming, Ascl1 displays pioneer activity by accessing and binding nucleosomal DNA as a single factor. Ascl1 is then able to actively recruit other transcription factors to many of its targets. Following on this, the study published in 2015 showed that Ascl1 functions also by promoting chromatin accessibility in its

native context, neurogenesis. Altogether, these studies give us clues as to how Ascl1 regulates its targets genes during adult neurogenesis in the hippocampus. Differential enhancer binding as well as differential transcription factor recruitment by Ascl1 are likely to regulate gene expression in the many Ascl1-dependent processes.

7.3 Biological significance of *Ascl1* function

Stem cells are a long-lived population of cells that are uniquely able to both self-renew and give rise to multiple cell types (Weissman, 2000). A number of adult tissues in the body, including the adult nervous system, retain the ability to regenerate cells lost to turnover, injury and/or disease throughout life thanks to the presence of populations of tissue-specific stem cells. Our work shows that *Ascl1* is crucial for the normal functioning of stem cells in the adult hippocampus.

In thinking about the role of *Ascl1* in the bigger picture, it is necessary to reflect on the function of neurogenesis in the adult brain. One aspect of this question relies on thinking about the role that the newly generated neurons play in hippocampal circuits; but maybe more relevant is to think how does stem cell activity and neurogenesis contribute to the general homeostasis of the adult brain. Within this topic, there are two main things to explore, and these are two sides of the same coin: how does the balance between self-renewal and quiescence confer such a unique status to tissues stem cells, and how their misregulation leads to aberrant phenotypes and disease. In this section I will very briefly discuss each of these topics and point out how the present work may fit within the broader framework of tissue stem cell biology.

7.3.1 *Ascl1* controls the balance between quiescence and self-renewal

Two central features define adult or tissue stem cells: they are able to self-renew and they give rise to differentiated progeny in response to the needs of the organism. Importantly, a number of tissues do this while being mostly in a dormant or quiescent state. From these features, two important points can be drawn: 1) it is the balance between stem cell self-renewal and quiescence that is responsible for tissue homeostasis, and 2) adult stem cells, not only rely on intrinsic control, but are also

able to listen to and, are therefore under, the control of their cellular environments (Nakada et al., 2011).

A number of models exist that aim to explain how the balance between quiescence and activation of stem cells in adult niches is regulated. Li and Clevers (2010), for example, propose the coexistence of distinct but adjoining pools of quiescent and active stem cells. In this "zoned" model of stem cell maintenance and self-renewal, which is opposed to a model where a single population of stem cells exists, quiescent stem cells function as a reserve population that is activated to replenish the active stem cells, or to regenerate tissue that has been lost or damaged. In the hematopoietic system, for example, a population of dormant hematopoietic stem cells (HSCs) reversibly switches from a quiescent to a self-renewing state at the interface between homeostasis and repair (Wilson et al., 2008). Rezza and colleagues (2014), on the other hand, propose that while rapidly renewing tissues like the bone marrow, the small intestine and the hair follicles in the skin have distinct stem cell populations as explained in the zoned model above, organs that undergo slow rates of cell turnover like the brain, muscle and liver, contain only relatively-quiescent or slow-dividing stem cells. These also maintain tissue homeostasis and can be activated upon injury, but they exist as a single population.

In the DG of the adult brain, due to a lack of stem cell markers, it is still unclear how many stem cell populations exist. Stem cells in the DG, as opposed to other stem cell niches where active and quiescent cells are differentially located facilitating the reception of distinct signals, do not show any apparent divergence in localisation that would allow them to form separate pools. Nonetheless, some evidence, like differential proliferative and neurogenic potentials observed with different cre lines (Bonaguidi et al., 2011, Bonaguidi et al., 2014 Sfn poster presentation), support the possibility that active and quiescent hippocampal stem cells also represent distinct pools and that the transition between the two is limited to very specific scenarios, like injury and/or disease (reviewed in Giachino and Taylor, 2014). This would be consistent with the observation that, while IPCs and neuroblasts are under constant external regulation, RGLs are rarely modulated by their environment (see section 1.3.4). The few examples that exist so far are epileptic seizures and acute social stress.

Interestingly, some of our results might also support the existence of separate populations of RGLs in the DG. For example, we find a much bigger proportion of stem cells expressing the early G_1 marker Ccnd1 compared to any other proliferation marker. Can this be marking a population of stem cells that are primed to be able to respond to proliferative signals, and therefore a different population from the deeply quiescent stem cells? Moreover, deletion of *RBPJκ* in the adult DG resulted in the initial activation of only approximately 40% of the stem cells. But what about the other 60% of stem cells? Can these represent the population that Li and Clevers (2010) consider to be deeply quiescent and therefore a back-up pool that only becomes activated after the loss of the active pool? These questions will certainly be addressed in the future by examining the fate and expression pattern of these putative populations in the long-term.

In any case, it is generally accepted that it is the niche that is responsible for inducing a quiescent versus active state in stem cells. In that way, quiescent and active stem cell populations will respond to corresponding inhibitory and stimulatory signals (Li and Clevers, 2010, Orford and Scadden, 2008, Rezza et al., 2014). These signals in the DG are, generally speaking, but with the exception of neuronal activity, which is not only unique but also very important in the adult brain, the same when compared to other adult stem cell niches (Rezza et al., 2014). Wnt, for example, is a potent mitogenic factor across a number of different tissues. On the other hand, BMP is well known to actively inhibit proliferation and induce quiescence. And as an added mechanism of control, the progeny of stem cells in many adult organs is thought to participate in a negative feedback involving Notch signalling to modulate the balance between self-renewal and quiescence.

It seems almost impossible that only one factor has such a central role in any process. The finding that Ascl1 is also key in the proliferation of RGLs in the SVZ in the adult brain only reinforces this conundrum (Andersen et al., 2014). It is also of interest the apparent conclusion that most of the signals described above act by regulating Ascl1 expression, either at the mRNA or protein level. In the future, it will be important to determine whether there is a factor playing such a central role in the control of stem cell activity in other adult tissues as *Ascl1* does in the adult brain, or if this niche is somehow unique. There is one factor whose role is reminiscent of that

of *Ascl1* and that is *MyoD* in muscle satellite cells. MyoD controls the transition of quiescence to activation in adult myogenic stem cells by regulating *Cdc6*, a component of the pre-replication complex (Zhang et al., 2010). Interestingly *Cdc6* was found to be a target of Ascl1 by ChIP-seq (see Table A1 1 in Appendix 1), but mRNA levels were found to be too low for its validation (not shown).

Going back to thinking about the bigger picture we ask ourselves, what does *Ascl1*'s role in the adult brain tells us about the homeostatic control of hippocampal neurogenesis? In the first place, it tells us that regulation is key for correct neurogenesis to take place. But it might also reveal some information regarding the evolution of this process. With having only one factor responsible for overseeing the huge task of generating enough neurons to be incorporated into the appropriate circuits of the hippocampus one might ask if this might not be a relatively new adaptation or late-evolved trait of mammals that confers adaptability to new challenging environments as suggested by Gerd Kempermann (Kempermann, 2012; see also section 1.1.4). Would the same principle be true for humans? Or is it possible that, as already discussed in previous sections, an alternative mechanism exists, and we just have not uncovered it? These are all interesting questions, and their answers may come in due course.

7.3.2 Misregulation of *Ascl1* may lead to disease

The importance of Ascl1 in the neurogenic process in the adult brain comes into focus when we think of the consequences of tilting the balance to either side. Loss of the self-renewal capacity and/or increased stem cell quiescence underlie both the natural process of ageing as well as some neurological disorders. On the other hand, over-proliferation of the stem cell compartment predisposes the tissue for cell overgrowth and cancer. There is, therefore, a constant need for evaluating the requirements of the tissue against the potential harms.

Our mouse model highlights the importance of maintaining a relatively quiescent population of cells in order to preserve the self-renewal and regenerative potential of the brain over time. A number of reports across the stem cell field now suggest that proliferation, and non self-renewing stem cell divisions, can lead to the

exhaustion of the stem cell pool (Encinas et al., 2011, Encinas and Sierra, 2012, Orford and Scadden, 2008). And this process, in part, underlies the age-related decline in adult hippocampal neurogenesis. Analysis of *Ascl1*-mutant mice five months after deletion revealed that the number of stem cells remains constant if no proliferation takes place, which is expected if the model above is correct (see section 1.3.4.1 for a discussion of ageing and neurogenesis).

The ageing process, nevertheless, seems to not only rely on an exhaustion of the stem cell population, but also on an increased quiescence of the remaining stem cells, which is mediated by both intrinsic and extrinsic mechanisms. An increase in the cyclin-dependent kinase inhibitor $p16^{INK4a}$ in neural progenitors in the SVZ during ageing corresponded with decreased neurogenesis (Molofsky et al., 2006). At the same time, increasing levels of quiescence-inducing factors with age negatively regulate stem cell activity. For example, accumulation of the Wnt antagonist Dkk1 in the DG during old age is suggested to be in part responsible for the reduction of neurogenesis and the decline in cognitive function in mice (Seib et al., 2013). This modulation of quiescence with increasing age could in principle be also mediated by regulation of Ascl1 expression. We indeed observe a reduction in the number of cells expressing Ascl1 over time (not shown). Quantification of these numbers, as well as of the levels of Ascl1 expression per cell will shed light on the process of age-related decline of neurogenesis observed in the DG.

On the other side of the balance, we find the role of Ascl1 in stem cell function to also have implications in the cancer field. High levels of Ascl1 expression were found in a number of neuro-endocrine tumours, including small cell lung carcinoma and prostate tumours (Ball, 2004, Vias et al., 2008, Augustyn et al., 2014), as well as several types of gliomas (Phillips et al., 2006, Somasundaram et al., 2005). In glioblastoma cancer stem cells, Ascl1 was also found to be important for the maintenance of tumorigenicity (Rheinbay et al., 2013). The finding that Ascl1 controls a number of cell cycle-related genes together with evidence of Ascl1 expression in tumours might suggest that these are causally related. Dysregulation of genes or pathways involved in important aspects of normal development, like growth, cell division and differentiation, is a way for precancerous cells to progress

to full tumorigenicity, and upregulation of Ascl1 appears to be one common mechanism by which tumours reach their state.

Put together, this evidence highlights the relevance of our results in the field of stem cell biology, since it is on this light that Ascl1 becomes key to the process of neurogenesis and that understanding how it is regulated becomes a central target for future treatments.

7.4 Future directions

Our work has addressed an important question in the field of adult neurogenesis: how do stem cells in the hippocampus integrate signals from the environment? However, many questions still remain unanswered. I have pointed out some of these throughout the text above, and I have provided an insight of possible directions to take when appropriate. There are, nonetheless, a few points that I believe deserve a more comprehensive discussion, and I aim to explain them below.

The first obvious question to ask is: how is Ascl1 regulated? In the first part of this chapter I discussed at length how different signals and transcription factors might interact with Ascl1 to modulate its expression at both the transcriptional and post-transcriptional levels. Most of these interpretations, however, are made by analogy with other systems. The lack of stem cell-specific markers, as well as the sensitivity of the system to change and regulation, make this topic a difficult one to tackle. An alternative to studying how Ascl1 and stem cell activity are regulated *in vivo* is the use of hippocampus-derived stem cell cultures. Isabelle Blomfield, a PhD student in the laboratory has taken this approach. She is using AH-NSCs and exposing them to BMP4, which is able to induce a quiescent-like state in them (Martynoga et al., 2013). In this way she can study the action of a number of signals normally present in the hippocampal neurogenic niche and how they function to fine-tune the quiescence versus activation balance. With this approach it will also be possible to assess Ascl1 expression and how it is affected under these different conditions.

We have focused our target analysis in genes related to the cell cycle. This was a decision based on the phenotype we observed and on the roles that we know Ascl1 plays in the ventral telencephalon. With this knowledge, however, the question of whether Ascl1 might not be playing other cell cycle-independent roles in hippocampal stem cells arises. The transition from quiescence to activation involves many cellular changes, like changes in lipid and oxygen metabolism, and changes in cell adhesion. These are possible cellular functions where Ascl1 could be important. In support of this idea, GO terms not related to the cell cycle are found as some of the top hits among the Ascl1-bound genes (see Figure 6-1). A short qPCR screen of transcripts of some of these genes in *WT* and *Ascl1^{neo}cKO* FACS-isolated hippocampal stem cells showed no significant differences between them (see Figure A1 4 in Appendix 1). In the future, it will be of interest to perform an unbiased transcriptomic analysis using RNA-Seq. In this way it will be possible to ascertain whether other genes that are bound by Ascl1 in AH-NSCs, are also misregulated in *Ascl1^{neo}cKO* mice, and are therefore direct targets, and ultimately establish whether *Ascl1* is involved in regulating genes outside of the cell cycle.

A topic that has come up throughout the discussion of this work is the possible existence of heterogeneity among the total pool of stem cells. Within this topic, a few clarifications are needed. Mainly, what kind of heterogeneity are we talking about? In the "zoned" model of stem cell maintenance and self-renewal proposed by Li and Clevers (2010) the two separate populations are differentiated by their being located in either activation- or quiescent-inducing environments within the niche. In such case, the two populations do not need to be developmentally different; their properties are induced by the signals they are exposed to, and their roles could potentially be swapped. Another scenario can be envisaged where the active and quiescent populations are different due to intrinsic differences that arise from, for instance, them developing from different progenitor pools. This idea could correspond with the proposal of the existence of a deeply quiescent pool of stem cells, which would be less responsive to external stimulation and would only become activated after an insult to the brain or after disease.

Yet, within this foundation, further subpopulations of stem cells might be described. And these distinctions can be on the basis of how primed they are for

activation or how neurogenic they are. All of these are active areas of research in the field at the moment. Our results, together with results from Noelia Urbán in the laboratory, point to the possibility that different populations of stem cells in the hippocampus do exist. Further characterisation of these cells and their behaviour over time will be required in the future to answer these questions. Moreover, Stefania Vaga, another postdoctoral fellow in the laboratory, will be taking on the challenge of carrying out single-cell RNA-Seq analysis of RGLs isolated from the adult hippocampus, an approach that will shed light on their potential heterogeneity.

Finally, and now moving into a systems perspective, I would like to discuss the implications of our results and our mouse model in the future of DG functional studies. The study of the role of new-born neurons in the adult hippocampus in cognitive performance has relied, on its most part, on several methods of ablation of neurogenesis. Such methods have included, among others, the administration of anti-mitotic drugs and irradiation (Deng et al., 2010). These approaches, however, have severe side effects in animals and the consequences of these on hippocampal function are not normally taken into consideration. Conditional *Ascl1* mutant mice could now be used as an alternative approach to these methods of neurogenesis ablation. Our animals show a complete block of neurogenesis, and even though not directly quantified, cell death does not appear to be a major event. Behavioural studies using both *Ascl1*^{neo}*cKO* and *Ascl1cKO* mice will provide important insights on the relevance of new neurons in the adult hippocampal circuit. We are currently collaborating with Nora Abrous, at the Neurocentre Magendie in Bordeaux, to perform these experiments.

7.5 Conclusions

Our study of the function of the proneural factor Ascl1 in the neurogenic lineage of the adult hippocampus has revealed a fundamental player in the control of stem cell activity. Ascl1 was exposed as an intermediate factor in stem cells connecting the outside world with the inner operative system of the cells to regulate neurogenic output and, ultimately, homeostasis (Figure 7-4).

Neurogenesis in the adult hippocampus is an extremely intricate and delicate process, with every step being influenced by innumerable extrinsic and intrinsic factors. Importantly, this regulation observed along the neurogenic lineage, was also shown to affect cognitive performance. We have provided evidence that Ascl1 is up- or downregulated in stem cells upon the reception of neurogenic or anti-neurogenic stimuli, respectively. Ascl1 directly regulates a number of cell cycle genes, among which the cyclin D factors are of huge importance, to control the activation of stem cells. Loss of Ascl1 completely abolishes precursor proliferation and, therefore, neurogenesis.

Our results are significant because this is the first transcription factor shown to act as an integrator of extrinsic signals in the adult brain. Our results are also partly unexpected. It is not common in nature to find only one factor having such a central role in one process. Even reduced levels of expression of *Ascl1* have an impact on the total neurogenic yield, emphasising the importance of correct Ascl1 regulation at both the protein and RNA levels to take place.

Recent years have seen a growth in the interest for exploiting the brain's endogenous reservoir of stem cells as a potential therapeutic strategy for tackling neurological disorders. In order for this to be possible it is necessary to understand what it is that enables stem cells to proliferate, and what mechanisms can be employed to extrinsically stimulate their proliferation and neuronal differentiation (Taylor et al., 2013). Our work has majorly contributed to the basic understanding of stem cell activation and proliferation, and on this basis will, hopefully, contribute to future therapies.

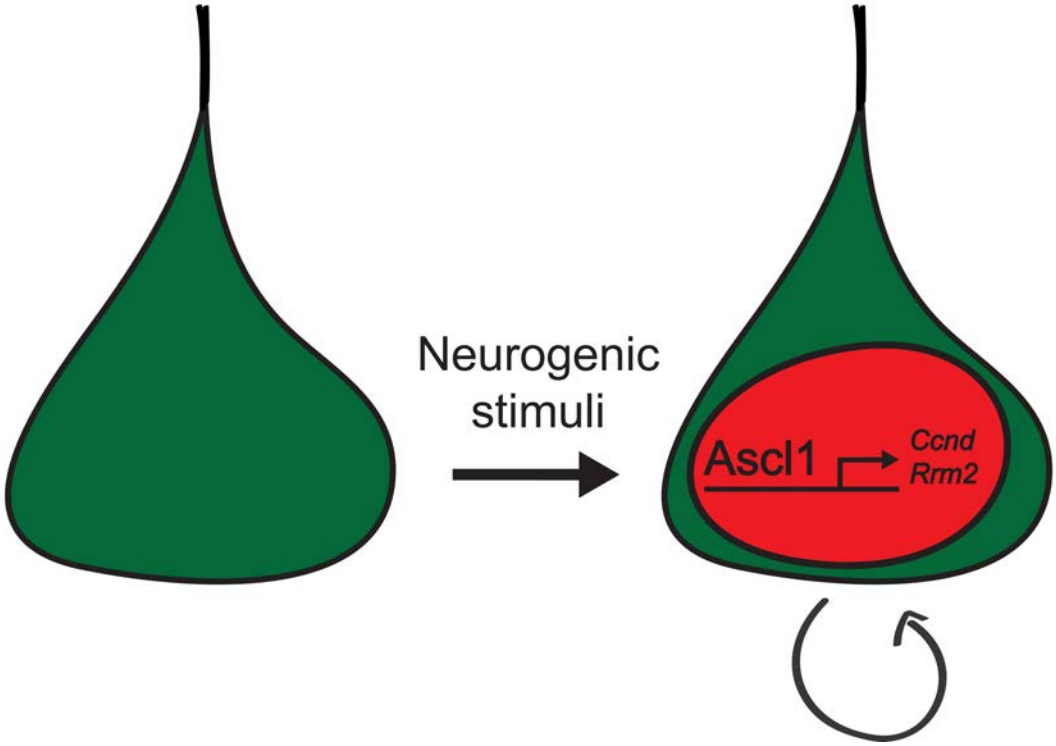

Figure 7-4 Model of Ascl1 function in adult hippocampal stem cells

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Appendix 1

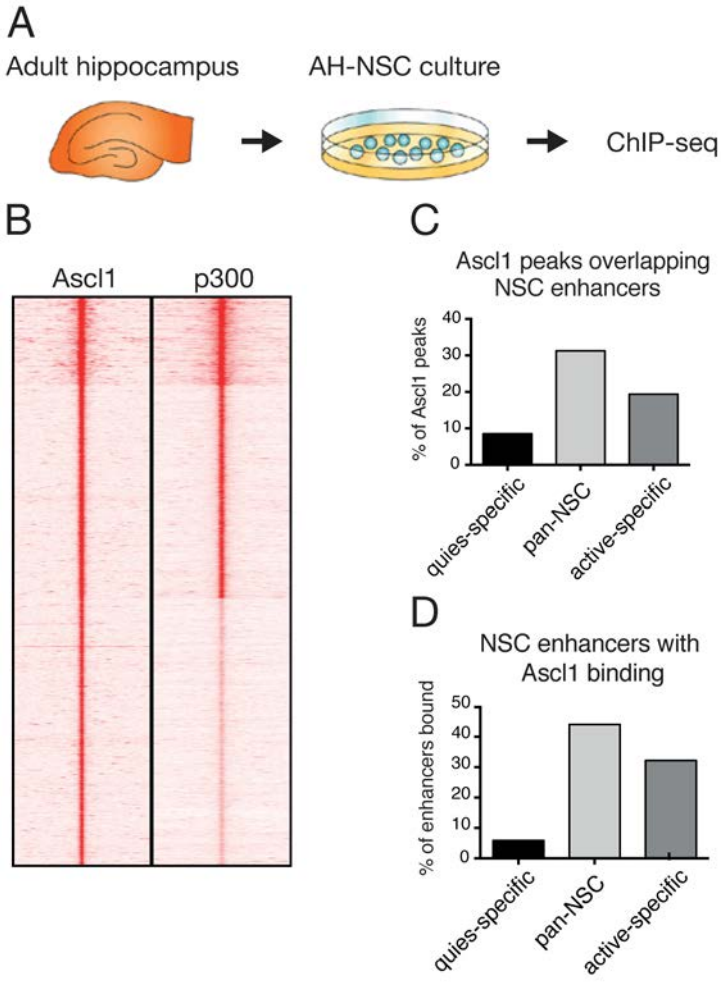

Figure A1 1 Genome-wide investigation of Ascl1 binding in AH-NSCs

(A) Diagram illustrating the derivation of adult hippocampus-derived NSC (AH-NSC) cultures to perform chromatin immunoprecipitation-sequencing (ChIP-seq). **(B)** Heat map representation of the density of ChIP-seq reads for Ascl1 in AH-NSCs and p300 marking enhancers in proliferating NSCs at the same genomic locations (from Martynoga et al., 2013) shows that a large part of Ascl1-binding sites are located in enhancers present in proliferating NSCs. **(C)** Similarly, the distribution of Ascl1-binding sites among different types of enhancers (from Martynoga et al., 2013) shows that a majority of Ascl1-binding sites map to enhancers previously identified in NSCs and that a big proportion of them are active-specific. **(D)** And, in a reciprocal manner, a large fraction of active-specific enhancers in NSCs are bound by Ascl1. quies-specific and active-specific are enhancers that are specific for quiescent or active NSCs, respectively. pan-specific enhancers are those that are present in both quiescent and active NSCs. For more information see Andersen et al., 2014 and Martynoga et al., 2013. Analysis performed by Ben Martynoga.

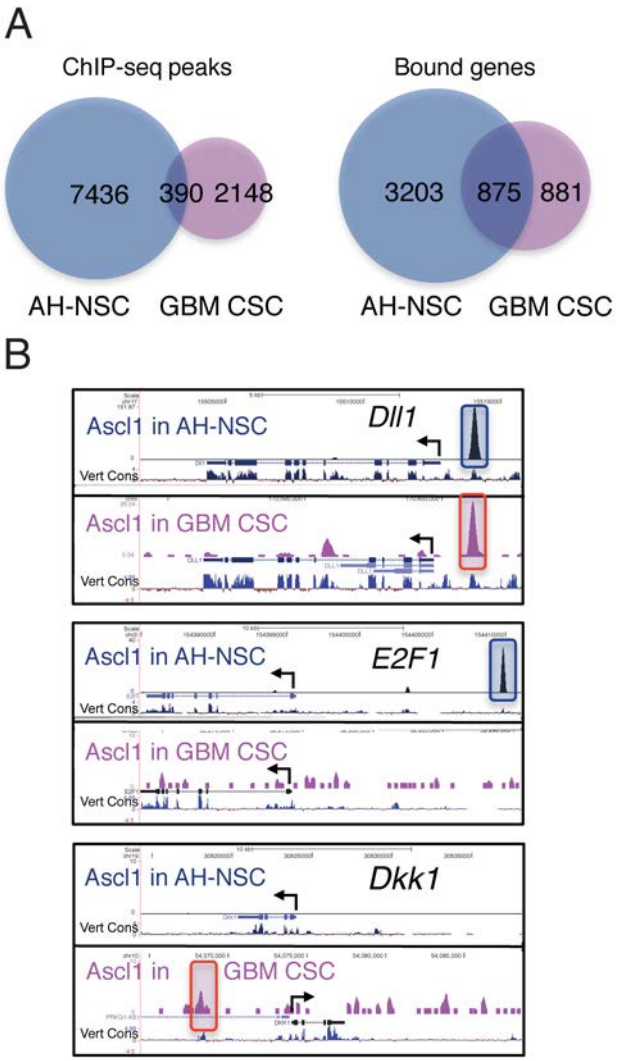

Figure A1 2 Comparison of Ascl1-bound genes in AH-NSCs and GBM CSCs

(A) Venn diagrams showing (left) the overlap between Ascl1 ChIP-seq peaks found in AH-NSCs (blue) and those found in glioblastoma cancer stem cells (GBM CSCs, pink, from Rheinbay et al., 2013), and (right) the overlap between Ascl1-bound genes in these two data sets. The overlap between these two data sets is not big. **(B)** Examples of ChIP-seq signals for Ascl1 in both AH-NSCs and GBM-CSCs. A binding peak is found for both cell types in the known Ascl1 target Dll1. For other genes, like E2f1, a peak is observed only in AH-NSCs; and yet for others, like Dkk1, a peak is found only in GBM-CSCs, suggesting that Ascl1 regulates different genes in different cell types. Analysis performed by Ben Martynoga.

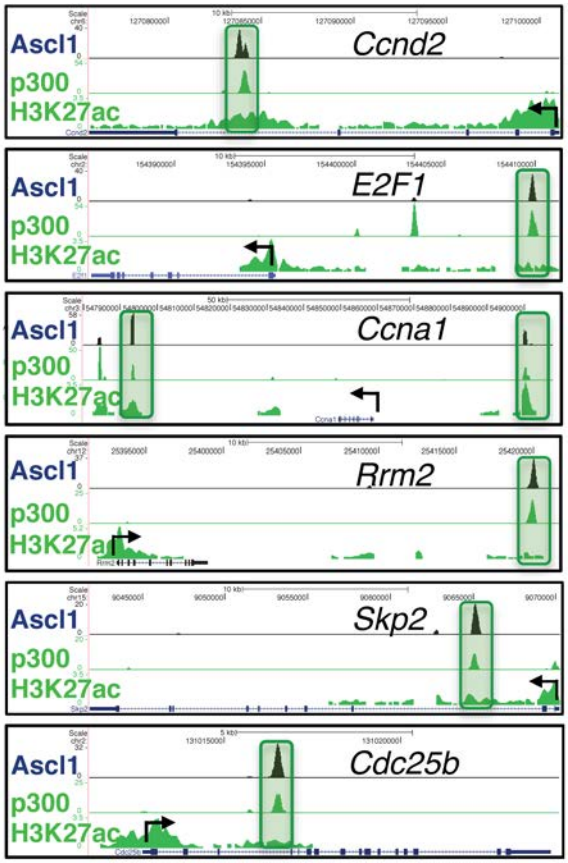

Figure A1 3 Cell cycle genes feature Ascl1-binding events

Examples of cell cycle-related genes that are bound by Ascl1 in AH-NSCs. For each gene the signal for Ascl1, and the enhancer marks for p300 and H3K27ac are shown (from Martynoga et al., 2013). Significant binding peaks are highlighted with a green box. The direction of transcription is indicated with an arrow. Analysis performed by Ben Martynoga.

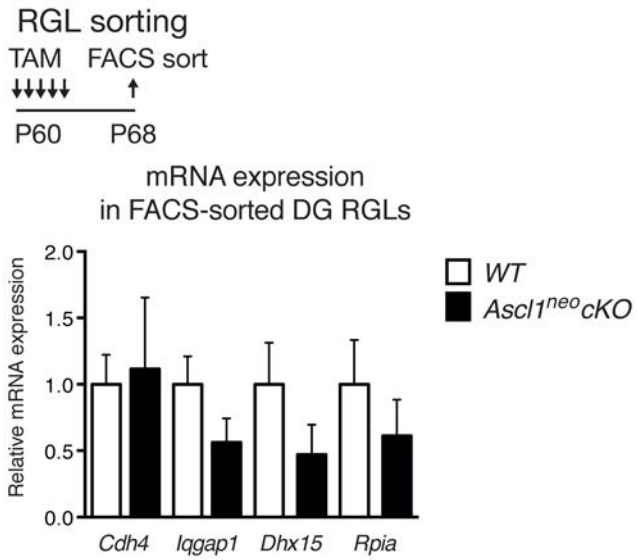

Figure A1 4 Non-cell cycle genes might also be regulated by Ascl1

Short screen to test whether other genes that are bound by Ascl1 but are not cell cycle-related are also misregulated in *Ascl1^{neo}cKO* mice. *Cdh4* is a cell-cell adhesion glycoprotein that is important in neuronal outgrowth; *Iqgap1* interacts with components of the cytoskeleton and with cell adhesion molecules to regulate cell morphology; *Dhx15* is a pre-mRNA processing factor involved in the disassembly of spliceosomes after the release of mature mRNA; *Rpia* is important in carbohydrate metabolism. In this case none of the genes are downregulated after *Ascl1* deletion. In the future, however, it will be of interest to examine this possibility more closely.

Table A1 1 Ascl1-bound genes in AH-NSCs

Gene name				
0610007P08Rik	Ckmt2	Gpr126	Noxa1	Sipa1l3
0610007P14Rik	Clasp2	Gpr146	Npas3	Sis
0610010O12Rik	Clasrp	Gpr149	Npc2	Six2
0610011L14Rik	Cldn11	Gpr15	Npepl1	Skap2
0610012H03Rik	Cldn23	Gpr153	Npff	Ski
1110002B05Rik	Cldn4	Gpr155	Npffr1	Skor1
1110012J17Rik	Clec14a	Gpr156	Npffr2	Skp2
1110021J02Rik	Clec2d	Gpr22	Npl	Slc10a4
1110028C15Rik	Clec2i	Gpr26	Nppa	Slc10a7
1110032A04Rik	Clec3b	Gpr3	Nptx1	Slc12a2
1110034B05Rik	Clec9a	Gpr30	Npvf	Slc12a3
1110038F14Rik	Clic1	Gpr50	Nqo1	Slc12a8
1110051M20Rik	Clic4	Gpr52	Nr1d1	Slc13a3
1110065P20Rik	Clic5	Gpr56	Nr2c1	Slc13a4
1110067D22Rik	Clic6	Gpr6	Nr2c2	Slc14a1
1190002N15Rik	Clip1	Gpr61	Nr2c2ap	Slc14a2
1300002K09Rik	Clip2	Gpr63	Nr2f1	Slc16a1
1500001M20Rik	Clip3	Gpr82	Nr2f2	Slc16a7
1500009L16Rik	Clip4	Gpr83	Nr2f6	Slc16a9
1500012F01Rik	Clmn	Gpr88	Nr3c1	Slc19a3
1500015O10Rik	Cln8	Gprc5b	Nr3c2	Slc1a1
1600012P17Rik	Clpb	Gprin2	Nr4a3	Slc1a2
1600014K23Rik	Clrn1	Gpsm1	Nr6a1	Slc1a3
1700001O22Rik	Clrn3	Gpt2	Nrbf2	Slc1a4
1700003F12Rik	Clvs1	Gramd1b	Nrcam	Slc1a6
1700008A04Rik	Cml2	Gramd2	Nrk	Slc22a12
1700010I14Rik	Cmpk2	Gramd3	Nrn1l	Slc22a16
1700010M22Rik	Cmtm5	Gramd4	Nrp1	Slc22a2
1700011E24Rik	Cmtm6	Grasp	Nrp2	Slc22a23
1700011H14Rik	Cngb1	Grb10	Nrsn1	Slc22a3
1700011I03Rik	Cnih4	Grb14	Nrxn1	Slc24a1
1700012B07Rik	Cnksr3	Grb2	Nrxn2	Slc24a2
1700016C15Rik	Cnn3	Greb1	Nrxn3	Slc24a3
1700016D06Rik	Cnnm3	Grem2	Nsg2	Slc24a5
1700017B05Rik	Cno	Grhl1	Nsmaf	Slc25a13
1700019G17Rik	Cnot2	Grhl2	Nsmce2	Slc25a18
1700019O17Rik	Cnot6l	Gria2	Nsun3	Slc25a24
1700020L24Rik	Cnp	Gria4	Nsun7	Slc25a29
1700023I07Rik	Cntn1	Grid2	Nt5dc3	Slc25a31
1700024P04Rik	Cntn4	Grik1	Ntm	Slc25a34
1700025G04Rik	Cntn5	Grin2b	Ntn1	Slc25a36
1700029H14Rik	Cntn6	Grin3a	Ntng1	Slc25a4
1700034H14Rik	Cntnap1	Grinl1a	Ntrk2	Slc25a40
1700034J05Rik	Cntnap4	Grip1	Ntrk3	Slc25a42
1700057G04Rik	Cntrob	Grk5	Ntsr2	Slc25a43
1700057K13Rik	Cobll1	Grlf1	Nuak1	Slc25a5
1700061J05Rik	Cog6	Grm1	Nuak2	Slc27a1
1700066M21Rik	Col12a1	Grm3	Nudt18	Slc27a6
1700090G07Rik	Col15a1	Grm4	Nudt2	Slc29a4
1700094C09Rik	Col18a1	Grm5	Nudt4	Slc2a10
1700102P08Rik	Col1a1	Grtp1	Nudt7	Slc30a10
1700106J16Rik	Col27a1	Grxcr1	Nudt9	Slc30a5
1700108M19Rik	Col2a1	Gsdmc	Nuf2	Slc30a8
1700110M21Rik	Col3a1	Gse1	Nup214	Slc31a2

Gene name				
1700112E06Rik	Col4a1	Gsg1l	Nup35	Slc35b1
1700129C05Rik	Col4a3	Gstm1	Nusap1	Slc35c2
1810011O10Rik	Col4a4	Gsto2	Nwd1	Slc35f1
1810020D17Rik	Col5a1	Gstt4	Nxnl2	Slc35f3
1810041L15Rik	Col5a3	Gstz1	Nxph1	Slc37a2
1810046K07Rik	Col8a1	Gsx1	Nynrin	Slc38a1
1810074P20Rik	Col9a1	Gtdc1	Nyx	Slc38a10
2010111I01Rik	Col9a3	Gtf2a1	ORF63	Slc38a3
2010317E24Rik	Colq	Gtf2i	Oaz2-ps	Slc38a6
2210009G21Rik	Commd9	Gtf3c4	Ociad1	Slc38a9
2210021J22Rik	Cops8	Gtpbp2	Odam	Slc39a11
2310035K24Rik	Coq2	Guca2a	Odc1	Slc39a12
2310046A06Rik	Corin	Gucy1b2	Odf3l1	Slc41a2
2310061N02Rik	Coro1c	Gucy1b3	Odz3	Slc41a3
2310067B10Rik	Coro2b	Gxylt1	Odz4	Slc44a1
2310079G19Rik	Cox18	Gypa	Ofcc1	Slc45a1
2410004B18Rik	Cox6c	H2-M10.1	Ogg1	Slc45a4
2410004P03Rik	Cox8c	H2-M10.3	Ola1	Slc48a1
2410066E13Rik	Cp	H2-M10.5	Olfm3	Slc4a3
2410131K14Rik	Cpa1	H2afy2	Olfml2a	Slc4a4
2610002I17Rik	Cpd	H60b	Olfml2b	Slc4a7
2610002J02Rik	Cpeb2	Haao	Olfml3	Slc5a7
2610021K21Rik	Cpeb4	Habp2	Olfr1324	Slc6a1
2610029G23Rik	Cplx2	Hadhb	Olfr1331	Slc6a11
2610039C10Rik	Cplx3	Hao1	Olfr1505	Slc6a12
2610109H07Rik	Cpm	Hap1	Olfr1513	Slc6a2
2610528E23Rik	Cpn2	Hapln1	Olfr161	Slc6a6
2700060E02Rik	Cpne3	Has2	Olfr24	Slc8a1
2810405K02Rik	Cpne7	Hbegf	Olfr412	Slc8a3
2810428I15Rik	Cps1	Hbp1	Olfr71	Slc9a6
2810474O19Rik	Cpxcr1	Hdac4	Olfr854	Slc9a9
2900010M23Rik	Cpxm1	Hdac5	Olig1	Slco1c1
2900011O08Rik	Cpxm2	Hdac7	Olig2	Slco3a1
2900026A02Rik	Crabp1	Hdac9	Olig3	Slit1
3110001D03Rik	Cradd	Hddc2	Oma1	Slitrk2
3110007F17Rik	Crb1	Hdgfl1	Opa1	Slitrk3
3110035E14Rik	Crb2	Hectd2	Opa3	Slmo1
3110056O03Rik	Creb3l1	Heg1	Opalin	Smad2
3110079O15Rik	Creb3l2	Helb	Oplah	Smad4
3300002A11Rik	Creb5	Hepacam	Oprd1	Smad5
3830431G21Rik	Crebbp	Hepacam2	Oprl1	Smad7
4632415K11Rik	Crhr2	Herpud2	Optc	Smarca2
4632428N05Rik	Crim1	Hes1	Orai1	Smarca5
4831426I19Rik	Crip1	Hes2	Orm1	Smc2
4833422F24Rik	Crip2	Hes6	Osbp2	Smg1
4921510H08Rik	Crispld2	Hexim1	Osbpl10	Smg6
4921511C20Rik	Crmp1	Hexim2	Osbpl5	Smoc1
4930402H24Rik	Crtam	Heyl	Osbpl8	Smoc2
4930403N07Rik	Cryab	Hfm1	Osbpl9	Smok2a
4930404H21Rik	Cryba4	Hgd	Osm	Smox
4930427A07Rik	Crybb1	Hhatl	Osmr	Smtn
4930449E01Rik	Crybb3	Hhipl1	Osr1	Smug1
4930452B06Rik	Crybg3	Hibadh	Osr2	Smyd2
4930453N24Rik	Cryz	Hic1	Osta	Smyd3
4930455C21Rik	Cs	Hic2	Otoa	Sncaip
4930467E23Rik	Csda	Hif1a	Otof	Snd1

Gene name				
4930468A15Rik	Csdc2	Hif1an	Otol1	Sned1
4930473A06Rik	Csk	Hip1	Otos	Snrk
4930474N05Rik	Csl	Hipk2	Otub1	Snrnp40
4930503L19Rik	Csmd2	Hist1h1b	Otud7b	Snrpe
4930506M07Rik	Csnk1a1	Hivep3	Otx2	Snta1
4930525F21Rik	Csnk1e	Hk1	Oxct1	Sntb1
4930549C01Rik	Csnk1g3	Hlcs	Oxr1	Sntn
4930562C15Rik	Csnk2a2	Hlx	Oxsm	Snx11
4930596D02Rik	Cspg4	Hmbox1	P2rx4	Snx20
4931440F15Rik	Cspg5	Hmcn1	P2ry1	Snx22
4932438H23Rik	Csrnp3	Hmcn2	P2ry12	Snx25
4933400A11Rik	Csrp1	Hmg20a	P2ry2	Snx29
4933403F05Rik	Csrp2	Hmga2	P2ry6	Snx30
4933407C03Rik	Cst3	Hmgb1	P4ha1	Snx5
4933409G03Rik	Cst6	Hmgcll1	P4ha2	Snx9
4933426M11Rik	Cst7	Hmgcr	Pabpc2	Sobp
4933427D06Rik	Cstad	Hnrnpu	Pabpc6	Socs3
4933430I17Rik	Ctbp2	Hnrpdl	Pacrg	Socs7
4933436I01Rik	Ctdp1	Hnrpll	Pacs2	Sod3
5031414D18Rik	Ctdspl	Homer2	Pacsin1	Sorbs2
5031439G07Rik	Ctgf	Hook1	Pacsin2	Sorcs1
5330437I02Rik	Ctnnb1	Hoxa1	Padi2	Sorcs2
5430435G22Rik	Ctnnbl1	Hoxa2	Pafah2	Sorcs3
5730403B10Rik	Ctnnd1	Hpcal1	Pag1	Sord
5730455O13Rik	Ctnnd2	Hrh1	Pak1	Sorl1
5730508B09Rik	Ctrb1	Hrh2	Pak6	Sos2
5730559C18Rik	Ctsc	Hrh4	Palm	Sox1
5830405N20Rik	Ctsl	Hs1bp3	Palmd	Sox10
5830433M19Rik	Ctso	Hs2st1	Pam	Sox11
6030405A18Rik	Cttnbp2	Hs3st3a1	Pamr1	Sox2
6030419C18Rik	Cubn	Hs6st1	Pank1	Sox21
6030446N20Rik	Cuedc1	Hsd17b3	Papd7	Sox3
6030498E09Rik	Cux1	Hspa8	Pappa	Sox4
6230409E13Rik	Cwc22	Hspb1	Pard3	Sox5
6330512M04Rik	Cx3cl1	Htr2c	Pard3b	Sox6
6330527O06Rik	Cxadr	Htr3a	Pard6b	Sox8
6430527G18Rik	Cxcl11	Htr6	Park2	Sox9
6530418L21Rik	Cxcl12	Htr7	Parm1	Sp3
8430427H17Rik	Cxcl13	Htra1	Parp11	Sp4
9030409G11Rik	Cxcr4	Htra4	Parp16	Sp5
9030418K01Rik	Cxcr7	Ibtk	Parvb	Sp8
9030617O03Rik	Cxxc4	Icos	Pax1	Spag9
9030625A04Rik	Cxxc5	Id1	Pax7	Sparc
9230019H11Rik	Cybrd1	Id2	Pax8	Sparcl1
9230109A22Rik	Cycs	Id3	Pbk	Spata13
9330159F19Rik	Cyld	Id4	Pbx1	Spata17
9430020K01Rik	Cyp24a1	Idh2	Pbx3	Spata19
9430031J16Rik	Cyp2d22	Ier3	Pcbp1	Spata21
9530068E07Rik	Cyp2j13	Iffo1	Pcca	Spata24
9630014M24Rik	Cyp2j9	Iffo2	Pcdh10	Spata3
9830001H06Rik	Cyp7b1	Ifnar1	Pcdh15	Spata6
9930013L23Rik	Cyr61	Ifnar2	Pcdh18	Spats2l
9930021D14Rik	Cysltr2	Ifngr1	Pcdh7	Specc1
A1bg	Cyth1	Ift27	Pcdh9	Speer2
A3galt2	Cyth4	Igdcc4	Pcgf5	Speer4d
A4galt	Cytip	Igf1	Pcnx	Spert

Gene name				
A530016L24Rik	Cytl1	Igf1r	Pcsk5	Spesp1
A530021J07Rik	D0H4S114	Igf2r	Pcsk6	Spg20
A630001G21Rik	D10Bwg1379e	Igfbp1	Pcx	Spg7
A630007B06Rik	D14Abb1e	Igfbp2	Pdcd1	Sphk1
A630055G03Rik	D15Ertd621e	Igfbp3	Pdcd6ip	Spns3
A630091E08Rik	D16Ertd472e	Igfbp4	Pde10a	Spo11
A730011L01Rik	D17Wsu104e	Igfbp5	Pde11a	Spock2
A830018L16Rik	D17Wsu92e	Igfbp7	Pde1a	Spon1
A930005I04Rik	D18Ertd653e	Igfbpl1	Pde1c	Spon2
A930011G23Rik	D19Bwg1357e	Igsf11	Pde2a	Spp2
A930018P22Rik	D19Ertd737e	Igsf21	Pde4b	Spred1
AA408296	D1Pas1	Igsf9b	Pde4d	Spry1
AI429214	D430041D05Rik	Ikbke	Pde5a	Spry2
AI467606	D5Ertd579e	Il12a	Pde9a	Spry4
AI480653	D6Wsu116e	Il13ra2	Pdgfa	Spsb4
AI593442	D8Ertd82e	Il17a	Pdgfc	Spty2d1
AI606181	D930014E17Rik	Il17b	Pdgfra	Spz1
AI646023	D930015E06Rik	Il17d	Pdgfrl	Sqle
AI837181	Daam1	Il17ra	Pdk4	Sqrdl
AK053790	Dab1	Il17rd	Pdlim1	Srbd1
AK085995	Dab2ip	Il18bp	Pdlim3	Src
AK132123	Dach1	Il23a	Pdlim5	Srcin1
AK168184	Dact1	Il33	Pdpn	Srebf1
AW146020	Dap	Il34	Pdss2	Srgap1
Aagab	Dapk1	Il3ra	Pdzd2	Srgap2
Aak1	Dapk2	Il9	Pdzd8	Srgap3
Aatk	Dapl1	Il9r	Pdzk1ip1	Srl
Abat	Dars	Impad1	Pdzrn3	Srp14
Abca1	Dbi	Impdh1	Pdzrn4	Srpk2
Abca13	Dbndd1	Inhbb	Pea15a	Srpx2
Abcb9	Dbx1	Ino80c	Pecam1	Srsf10
Abcc12	Dbx2	Ino80d	Peci	Srsf3
Abcc3	Dcaf5	Inpp4b	Pef1	Srsf5
Abcc4	Dcakd	Inpp5a	Peg12	Srsf7
Abcd2	Dcc	Inpp5f	Peli2	Srsf9
Abhd10	Dcdc2c	Inpp5k	Pemt	Ss18
Abhd2	Dclk1	Inppl1	Penk	Ssbp2
Abi2	Dclk2	Ins1	Pepd	Ssbp3
Abi3bp	Dclk3	Insc	Pet112l	Ssr3
Abl1	Dclre1b	Insig1	Pex5l	Sst
Ablim1	Dclre1c	Insig2	Pfkfb3	Sstr4
Ablim2	Dcn	Insm1	Pfn2	Sstr5
Abra	Dcp1b	Insr	Pga5	St18
Abtb2	Dcp2	Integrin Alpha-4	Pgap1	St3gal1
Acaca	Dctd	Intu	Pgf	St3gal2
Acacb	Dcx	Invs	Pgk1	St3gal3
Acadl	Ddc	Ip6k3	Pgm5	St3gal4
Acap2	Ddhd1	Ipo5	Pgpep1l	St3gal5
Acap3	Ddi1	Iqcj-schip1	Pgrmc1	St3gal6
Accn2	Ddit4	Iqgap1	Pgrmc2	St5
Accn4	Ddit4l	Iqsec1	Pgs1	St6gal2
Accn5	Ddr1	Ireb2	Phactr2	St6galnac3
Accsl	Ddr2	Irf2	Phc2	St6galnac5
Acer3	Ddx1	Irf2bp2	Phf12	St7
Acly	Ddx11	Irf4	Phf15	St8sia1
Acn9	Ddx18	Irf6	Phf17	St8sia2

Gene name				
Aco1	Ddx20	Irgm2	Phf20	St8sia3
Acot11	Ddx26b	Irs1	Phf21a	Stab2
Acot12	Ddx3x	Irs2	Phlda1	Stag1
Acox3	Ddx6	Irx1	Phldb1	Stambpl1
Acoxl	Dear1	Irx2	Phldb2	Stard13
Acpl2	Degs2	Irx3	Phlpp1	Stard3
Acsl3	Dennd2a	Irx5	Phox2b	Stard5
Acss1	Dennd4a	Irx6	Phtf1	Stard9
Acss3	Dennd5a	Islr	Phtf2	Stat1
Actb	Derl1	Itga11	Phyhd1	Stat3
Actbl2	Dgkb	Itga2	Phyhipl	Stat4
Actc1	Dgkd	Itgae	Pi4k2a	Stat5a
Actg1	Dgkg	Itgav	Pick1	Stc1
Actl7b	Dgki	Itgb1	Pid1	Steap3
Actn1	Dgkz	Itgb1bp2	Pigc	Stim2
Actn2	Dguok	Itgb2l	Pigv	Stk17b
Actr3	Dhrs2	Itgb3	Pigy	Stk24
Actr3b	Dhrs3	Itgb5	Pigz	Stk32a
Acvr1	Dhx15	Itgb6	Pik3c3	Stk32b
Acvr2a	Dhx30	Itgb8	Pik3ca	Stk32c
Acyp2	Dhx35	Itih5	Pik3cd	Stk33
Ada	Dhx36	Itm2b	Pik3cg	Stk35
Adam10	Dhx40	Itm2c	Pik3r1	Stk38l
Adam12	Dhx8	Itpkb	Pim3	Stmn2
Adam19	Dhx9	Itpr1	Pin1	Stmn4
Adam30	Diap1	Itpr2	Pinx1	Stox2
Adam33	Diap2	Itpripl2	Pion	Strap
Adamts12	Dicer1	Itsn2	Pip4k2a	Stt3b
Adamts14	Dio2	Ivns1abp	Pipox	Stx8
Adamts16	Dip2b	Iyd	Pirt	Stxbp1
Adamts17	Dip2c	Jag1	Pitpnb	Stxbp3a
Adamts18	Diras2	Jak2	Pitpnc1	Stxbp4
Adamts20	Dirc2	Jakmip2	Pitpnm2	Stxbp6
Adamts4	Dis3l	Jam2	Pitrm1	Sucla2
Adamts5	Dis3l2	Jam3	Pja1	Suclg2
Adamts6	Disc1	Jarid2	Pkd1	Sulf1
Adamts7	Disp2	Jazf1	Pkd1l1	Sulf2
Adamts8	Dkk3	Jdp2	Pkd2l2	Sult5a1
Adamts9	Dleu7	Jhdm1d	Pkhd1	Sun1
Adamtsl1	Dlg2	Jmjd1c	Pkig	Susd1
Adamtsl2	Dlg5	Jmy	Pkm2	Sv2b
Adamtsl3	Dlgap1	Josd2	Pkn2	Sv2c
Adarb1	Dlgap3	Jph1	Pknox1	Svil
Adarb2	Dlgap4	Jun	Pknox2	Swap70
Adc	Dll1	Jup	Pkp4	Syce1l
Adcy1	Dll3	Kalrn	Pla2g16	Sykb
Adcy7	Dlst	Kank1	Pla2g2c	Syn2
Adcyap1r1	Dlx2	Kank2	Pla2g7	Sync
Add2	Dmp1	Kank4	Plac9	Syne1
Adhfe1	Dmrt1	Kat2b	Plcb1	Syt1
Adk	Dmrtb1	Katnal1	Plcb4	Syt11
Adm	Dnahc10	Kbtbd11	Plcd4	Syt13
Ado	Dnahc14	Kbtbd13	Plce1	Syt3
Adora1	Dnahc5	Kbtbd8	Plcg1	Syt6
Adora2b	Dnaic2	Kcmf1	Plcl1	Syt8
Adora3	Dnajb12	Kcna10	Plcl2	Sytl2

Gene name				
Adra1a	Dnajb5	Kcnab2	Plcxd2	Sytl3
Adra1b	Dnajb8	Kcnc1	Pld1	T
Adra2c	Dnajb9	Kcnc4	Pld6	Tac1
Adrb1	Dnajc1	Kcnd2	Plekha1	Tacc1
Adrb2	Dnajc19	Kcnd3	Plekha5	Tacc3
Adrbk2	Dnajc28	Kcne4	Plekha7	Tacr3
Adssl1	Dnajc5b	Kcnh5	Plekhb1	Tada3
Aebp2	Dnase1l3	Kcnh7	Plekhf1	Taf12
Afap1	Dner	Kcnip2	Plekhf2	Taf4a
Afap1l2	Dnlz	Kcnip3	Plekhg3	Taf4b
Aff1	Dnm2	Kcnj10	Plekhg5	Tagln2
Aff2	Dnmt1	Kcnj12	Plekhh2	Tanc1
Aff3	Dnmt3b	Kcnj13	Plekhh3	Tanc2
Afm	Dntt	Kcnj2	Plekho1	Tank
Agap1	Dock1	Kcnj5	Plg	Tarbp1
Agbl1	Dock10	Kcnj6	Plk1s1	Tas2r119
Agmat	Dock2	Kcnk1	Plk2	Tas2r134
Agpat3	Dock3	Kcnk13	Plk3	Tatdn1
Agpat4	Dock4	Kcnk15	Pllp	Tax1bp1
Agr3	Dock7	Kcnk2	Plod2	Tbc1d1
Agrn	Dok5	Kcnk3	Plrg1	Tbc1d10a
Agtpbp1	Dok7	Kcnma1	Pls3	Tbc1d14
Agtr1a	Dolpp1	Kcnn2	Pltp	Tbc1d16
Ahcy	Dopey2	Kcnn3	Plxdc2	Tbc1d22a
Ahcyl1	Dpf3	Kcnn4	Plxna1	Tbc1d23
Ahdc1	Dpp10	Kcnq1	Plxna2	Tbc1d30
Ahi1	Dpp6	Kcnq3	Plxna4	Tbc1d7
Ahr	Dpp8	Kcnq4	Plxnd1	Tbcel
Ahsg	Dpyd	Kcnrg	Pmaip1	Tbl1x
Aig1	Dpysl2	Kcns2	Pmepa1	Tbl1xr1
Aim1l	Dpysl3	Kcnt1	Pmp2	Tbx1
Aim2	Dpysl5	Kctd1	Pmp22	Tbx19
Aipl1	Dscam	Kctd13	Pnlip	Tbx2
Ak2	Dscaml1	Kctd14	Pnma1	Tceal8
Ak4	Dscr3	Kctd16	Pnmal1	Tcf12
Akap10	Dst	Kctd18	Pnmal2	Tcf20
Akap13	Dstyk	Kctd21	Pnrc1	Tcf21
Akap17b	Dtl	Kctd3	Poc1a	Tcf4
Akap2	Dtna	Kctd8	Poc1b	Tcf7l1
Akap6	Dtnbp1	Kdelr2	Poc5	Tcf7l2
Akap7	Dtx4	Kdm2b	Pofut2	Tcfap2c
Akap8l	Duox2	Kdm4d	Pol	Tcp10a
Akna	Duoxa2	Kdm6b	Pola2	Tcp11
Akr1d1	Dupd1	Khdrbs3	Pold3	Tcra
Akt2	Dusp10	Kif13a	Polg	Tdrd6
Akt3	Dusp26	Kif13b	Polm	Tdrd7
Alcam	Dusp4	Kif14	Polr1a	Tead1
Aldh1a7	Dusp5	Kif16b	Polr2i	Tead4
Aldh1l1	Dusp6	Kif1b	Polr3b	Tecta
Aldh4a1	Dusp7	Kif20b	Polr3h	Tek
Aldh8a1	Duxbl	Kif21a	Polr3k	Tekt1
Aldoc	Dync1i1	Kif21b	Pom121	Tekt3
Alg10b	Dync1i2	Kif26a	Pon1	Tekt5
Alox12	Dynlrb2	Kif26b	Pop1	Tenc1
Alpl	Dynlt1c	Kif3a	Pou1f1	Tesc
Alx4	Dynlt3	Kif5c	Pou2af1	Tesk1

Gene name				
Amfr	Dyrk1a	Kifc3	Pou2f1	Tet2
Amot	E030019B06Rik	Kirrel3	Pou2f2	Tet3
Amotl1	E030025P04Rik	Kit	Pou3f1	Tex14
Amotl2	E130114P18Rik	Kitl	Pou3f2	Tex2
Ampd3	E130203B14Rik	Kl	Pou3f3	Tfb1m
Amph	E130309F12Rik	Klf10	Pou3f4	Tfdp2
Amz1	E2f1	Klf12	Pou4f1	Tff3
Anapc1	E2f3	Klf13	Ppap2b	Tgfb2
Anapc16	E2f7	Klf14	Ppapdc1a	Tgfb3
Angpt1	E2f8	Klf15	Ppara	Tgfbi
Angpt2	E330034G19Rik	Klf2	Pparg	Tgfbr1
Angpt4	Ear14	Klf3	Ppargc1a	Tgfbr2
Angptl4	Ebf1	Klf4	Ppargc1b	Tgfbr3
Ank	Ebna1bp2	Klf6	Ppcdc	Tgif1
Ank2	Ece1	Klf7	Ppef2	Tgif2
Ank3	Echdc3	Klf9	Ppfia1	Tgif2lx1
Ankib1	Edar	Klhdc5	Ppfibp2	Tgm2
Ankrd1	Edc3	Klhdc7a	Ppif	Tgoln2
Ankrd11	Edem1	Klhl13	Ppil1	Thada
Ankrd17	Edem3	Klhl23	Ppm1a	Thbs2
Ankrd23	Edil3	Klhl25	Ppm1e	Thbs4
Ankrd26	Edn2	Klhl29	Ppm1f	Thoc3
Ankrd28	Ednrb	Klhl32	Ppm1h	Thoc7
Ankrd40	Eea1	Klhl36	Ppm1l	Thra
Ankrd43	Eef1a1	Klhl38	Ppnr	Thrsp
Ankrd45	Eef1g	Klhl5	Ppp1cb	Thsd4
Ankrd46	Eef2k	Klhl6	Ppp1r10	Thumpd3
Ankrd55	Eefsec	Klhl8	Ppp1r12a	Thy1
Ankrd57	Efcab3	Kndc1	Ppp1r12b	Tiam2
Ankrd58	Efcab4b	Krc	Ppp1r14b	Ticam1
Ankrd6	Efcab5	Kremen1	Ppp1r14c	Tigit
Ano2	Efhd2	Krt8	Ppp1r16b	Timp3
Ano6	Efnb2	Krtap13-1	Ppp1r2	Timp4
Anp32a	Efr3a	Krtap26-1	Ppp1r3b	Tinag
Antxr1	Eftud1	Krtap5-4	Ppp1r3c	Tinagl1
Anxa1	Egfl6	Ky	Ppp1r9a	Tjp1
Anxa11	Egfl7	L1td1	Ppp1r9b	Tk1
Anxa2	Egflam	L3mbtl3	Ppp2ca	Tkt
Anxa5	Egfr	Lama4	Ppp2r1b	Tle1
Anxa6	Egln3	Lama5	Ppp2r2a	Tle3
Aoah	Ehbp1	Lamb1	Ppp2r2b	Tle4
Aox1	Ehd2	Lamb3	Ppp2r3a	Tll1
Ap3m1	Ehd4	Lamc1	Ppp2r5a	Tln2
Apaf1	Ehf	Lamc2	Ppp2r5c	Tlr1
Apba2	Eid1	Laptm4b	Ppp2r5e	Tlr5
Apbb2	Eif1b	Laptm5	Ppp5c	Tlx3
Apcdd1	Eif2b3	Lasp1	Ppyr1	Tm2d1
Apln	Eif2d	Lass5	Prcp	Tm4sf1
Apoa1	Eif3f	Lats2	Prdm10	Tm4sf19
Apobec2	Eif3h	Lbh	Prdm11	Tm6sf1
Apol11b	Eif4g3	Lbr	Prdm2	Tm9sf4
Apol6	Elac2	Lcat	Prdm5	Tmbim1
Apol8	Elavl2	Lclat1	Prdx6	Tmbim6
Aqp1	Elavl4	Lcp2	Prelid2	Tmc1
Aqp6	Elf2	Ldhal6b	Prep	Tmc2
Ar	Elf5	Ldlrad3	Prex1	Tmc3

Gene name				
Araf	Elfn1	Ldoc1l	Prf1	Tmc5
Arap2	Elfn2	Lect1	Prickle1	Tmc6
Arap3	Elk3	Lef1	Prkag2	Tmcc1
Areg	Ell2	Lemd1	Prkar1a	Tmcc2
Arfgap2	Elmo1	Lemd2	Prkar1b	Tmcc3
Arg1	Eln	Lep	Prkca	Tmco5
Arhgap12	Elovl4	Lfng	Prkcb	Tmco7
Arhgap15	Elovl6	Lgals1	Prkce	Tmed10
Arhgap18	Emid1	Lgals8	Prkd1	Tmeff1
Arhgap21	Emid2	Lgi1	Prkdc	Tmeff2
Arhgap22	Emilin3	Lgmn	Prkg2	Tmem100
Arhgap23	Eml1	Lgr4	Prl3c1	Tmem104
Arhgap24	Emp1	Lgr5	Prl5a1	Tmem108
Arhgap26	Emp3	Lhfp	Prlhr	Tmem117
Arhgap27	Emx2	Lhfpl1	Prmt8	Tmem120a
Arhgap31	Enc1	Lhfpl3	Proca1	Tmem131
Arhgap32	Enho	Lhx2	Prok2	Tmem132e
Arhgap39	Enox1	Lhx4	Prokr1	Tmem149
Arhgap42	Enpp1	Lhx8	Prom2	Tmem161b
Arhgef10l	Enpp5	Lif	Pros1	Tmem163
Arhgef18	Entpd1	Lifr	Prox1	Tmem164
Arhgef2	Epb4.1	Lima1	Prpf18	Tmem167
Arhgef3	Epb4.1l4b	Limch1	Prpf19	Tmem17
Arhgef37	Epha4	Limd1	Prpf4b	Tmem171
Arid1a	Epha5	Lime1	Prpsap2	Tmem173
Arid1b	Ephb1	Limk2	Prr15	Tmem174
Arid2	Ephb2	Lims1	Prr18	Tmem176b
Arid3a	Ephb3	Lin28a	Prr5	Tmem177
Arid3b	Ephx1	Lingo1	Prr5l	Tmem178
Arid4a	Ephx3	Lingo4	Prrc1	Tmem18
Arid5a	Eps8	Lipc	Prrc2b	Tmem182
Arid5b	Erc1	Lipg	Prss48	Tmem188
Arl11	Ercc2	Litaf	Prss52	Tmem194b
Arl2bp	Ercc4	Lix1	Prtg	Tmem196
Arl4a	Erf	Lmcd1	Psap	Tmem2
Arl4c	Ergic1	Lmf1	Psapl1	Tmem20
Arl6ip6	Erh	Lmln	Psat1	Tmem209
Armc1	Erlin2	Lmna	Psd3	Tmem211
Armc2	Erv3	Lmnb1	Psma4	Tmem215
Arnt2	Esm1	Lmo1	Psma6	Tmem22
Arntl	Esrrb	Lmo2	Psmb1	Tmem220
Arpp21	Esrrg	Lmo3	Psmc1	Tmem229b
Arrdc3	Esyt3	Lmo4	Psmc6	Tmem231
Arsb	Etaa1	Lmo7	Psmd7	Tmem232
Arsi	Etl4	Lmod3	Psmd9	Tmem38b
Artn	Etnk1	Lmx1b	Psmg4	Tmem39b
Asap1	Ets1	Lnp	Pstpip2	Tmem41b
Asap2	Ets2	Lnx1	Ptar1	Tmem44
Asap3	Etv1	Loh12cr1	Ptbp2	Tmem51
Asb4	Etv4	Lonrf1	Ptcd2	Tmem52
Asb6	Etv5	Lpar4	Ptch1	Tmem55a
Asb8	Etv6	Lpar6	Ptchd2	Tmem63a
Ascl1	Evc2	Lpcat1	Pten	Tmem64
Asf1a	Evi5	Lpcat2	Pter	Tmem66
Ash1l	Evi5l	Lpcat4	Ptgds	Tmem69
Asnsd1	Evpl	Lphn2	Ptges	Tmem72

Gene name				
Astn1	Evx1	Lphn3	Ptgfr	Tmem86a
Astn2	Exoc3	Lpin1	Ptgs2	Tmem87b
Asxl1	Exoc4	Lpl	Pth1r	Tmem90a
Atad2b	Exoc6b	Lpp	Pth2r	Tmem91
Ate1	Exog	Lrch1	Ptk2	Tmie
Atf1	Exosc9	Lrfn4	Ptk2b	Tmod1
Atf7	Exph5	Lrguk	Ptma	Tmod2
Atg10	Ext1	Lrig1	Ptms	Tmpo
Atg4c	Extl3	Lrig3	Ptn	Tmprss2
Atg7	Eya1	Lrp11	Ptp4a3	Tmsb10
Atl2	Eya2	Lrp12	Ptpdc1	Tmsb4x
Atoh1	F13a1	Lrp1b	Ptplad2	Tmtc2
Atoh8	F2rl1	Lrp4	Ptpn1	Tmtc4
Atp10a	F2rl2	Lrp5	Ptpn13	Tnc
Atp11a	F3	Lrp6	Ptpn3	Tnfrsf11b
Atp11b	F830045P16Rik	Lrp8	Ptpn4	Tnfrsf19
Atp13a4	Fabp2	Lrpap1	Ptpn5	Tnfrsf21
Atp1a1	Fabp7	Lrpprc	Ptpra	Tnfsf11
Atp1a2	Fadd	Lrrc28	Ptprb	Tnfsf13b
Atp1b2	Fads1	Lrrc29	Ptprc	Tnfsf15
Atp1b3	Fah	Lrrc3b	Ptprd	Tnfsf18
Atp2b1	Fam101a	Lrrc4	Ptprg	Tnik
Atp5g2	Fam101b	Lrrc40	Ptprj	Tnk2
Atp5g3	Fam102a	Lrrc42	Ptprm	Tnnt2
Atp5l	Fam102b	Lrrc4c	Ptprn2	Tnp1
Atp5o	Fam105a	Lrrc68	Ptpro	Tnr
Atp6ap1l	Fam107a	Lrrc8b	Ptprs	Tnrc6a
Atp6v0e	Fam108c	Lrrc8c	Ptprt	Tnrc6b
Atp6v1a	Fam110a	Lrrc8d	Ptprz1	Tnrc6c
Atp6v1e2	Fam114a1	Lrrc9	Ptrf	Tns1
Atp6v1g3	Fam120a	Lrrfip1	Pum1	Tns3
Atp8a1	Fam120b	Lrriq3	Purb	Tns4
Atp8b5	Fam122a	Lrrn1	Pus7	Tob2
Atp9a	Fam122b	Lrrn3	Pvrl1	Tom1l1
Atrnl1	Fam123a	Lrrtm2	Pwwp2a	Tom1l2
Atxn1	Fam124b	Lrrtm3	Pwwp2b	Tomm20
Atxn10	Fam125b	Lrtm1	Pxdn	Tomm40
Atxn2	Fam131a	Lrtm2	Pygl	Top1
Atxn7l1	Fam134b	Lsamp	Qk	Tox
Atxn7l3b	Fam135b	Lsm5	Qpct	Tox2
Auh	Fam13c	Lsm6	Qprt	Tox3
Aurkaip1	Fam154b	Lta4h	Qrfp	Tpcn1
Auts2	Fam160b1	Ltbp1	Qrsl1	Tpcn2
Aven	Fam167a	Ltbp4	Qsox2	Tpd52
Axin2	Fam173a	Ltc4s	RP23-125A1.2	Tpd52l1
Azi2	Fam173b	Lxn	Rab11a	Tpm1
Azin1	Fam174a	Ly6c2	Rab11fip3	Tppp
B020018G12Rik	Fam174b	Ly75	Rab11fip4	Tpra1
B230312A22Rik	Fam178b	Ly86	Rab11fip5	Tprg
B3galnt1	Fam180a	Lyn	Rab14	Tpst2
B3galt2	Fam181b	Lynx1	Rab17	Tpt1
B3galt5	Fam184b	Lypd1	Rab20	Tra2b
B3gat1	Fam185a	Lypd6	Rab32	Traf1
B3gnt2	Fam186b	Lyzl1	Rab3b	Traf3
B4galt1	Fam189a2	Lzts1	Rab3c	Traf3ip2
B4galt3	Fam18a	Lzts2	Rab3gap2	Traf3ip3

Gene name				
B9d1	Fam192a	Mab21l1	Rab4a	Traf4
BC017612	Fam198b	Mab21l2	Rab6b	Traf5
BC018507	Fam19a2	Macf1	Rab8b	Traf6
BC023829	Fam19a3	Macrod1	Rabgap1l	Trak1
BC024659	Fam19a4	Mad1l1	Rabggta	Tram1
BC026439	Fam19a5	Mael	Racgap1	Tram2
BC027231	Fam20b	Maf	Rad51l1	Trap1
BC030867	Fam20c	Maged1	Raet1d	Trappc3
BC031353	Fam38b	Magee1	Ralbp1	Trappc9
BC031781	Fam3b	Magi1	Ralgapa2	Trdn
BC032203	Fam3c	Magi3	Ralgds	Treh
BC034090	Fam43a	Magoh	Ralgps1	Trh
BC035947	Fam46a	Malt1	Raly	Trib1
BC037703	Fam49b	Maml2	Ramp1	Trib2
BC048546	Fam53b	Maml3	Ranbp3l	Trib3
BC048562	Fam55b	Man1c1	Ranbp6	Tril
BC052040	Fam55d	Man2a1	Rap1a	Trim16
BC057022	Fam58b	Manba	Rap1gap	Trim2
BC067074	Fam59a	Map1lc3b	Rap2b	Trim26
BC100451	Fam5b	Map2k5	Rap2c	Trim29
BC106179	Fam5c	Map2k6	Rapgef1	Trim3
Bace2	Fam64a	Map3k1	Rapgef2	Trim32
Bach2	Fam65b	Map3k13	Rapgef3	Trim33
Bag1	Fam69a	Map3k14	Rapgef4	Trim44
Bag3	Fam69c	Map3k7	Rapgef5	Trim52
Bag5	Fam70b	Map3k9	Rarb	Trim55
Bahcc1	Fam76a	Map4k3	Rars	Trim62
Bai2	Fam76b	Map4k4	Rasa3	Trim67
Baiap2	Fam78a	Mapk10	Rasal2	Trim71
Bank1	Fam78b	Mapk4	Rasgef1c	Trim8
Banp	Fam82a1	Mapk8	Rasgrf2	Trim9
Barhl1	Fam83a	Mapkap1	Rasgrp1	Trio
Barx2	Fam83f	Mapkapk2	Rassf10	Trit1
Baz2b	Fam84b	Mapre1	Rassf2	Trmt5
Bbox1	Fam86	Mapre2	Rassf3	Trmt61a
Bbs10	Fam89a	Mapre3	Rbfox1	Trmu
Bbx	Fam92a	Mapt	Rbfox2	Trp53i11
Bcan	Fam92b	March1	Rbl2	Trpc4ap
Bcar1	Fam98b	March3	Rbm15	Trpm1
Bcar3	Fancc	Marcks	Rbm19	Trpm3
Bcas1	Farp1	Mast2	Rbm20	Trps1
Bcat1	Fars2	Mast4	Rbm24	Tsc22d1
Bche	Fat1	Matn2	Rbm28	Tsc22d2
Bckdhb	Fat3	Max	Rbm47	Tsc22d3
Bcl11b	Fat4	Mb	Rbms3	Tsga14
Bcl2	Fbl	Mbnl1	Rbmxl2	Tsga8
Bcl2l11	Fbln1	Mbnl2	Rbpj	Tshr
Bcl6	Fbln2	Mboat2	Rc3h1	Tshz1
Bcl7a	Fbn2	Mbp	Rcbtb2	Tshz2
Bcl9	Fbp1	Mc2r	Rchy1	Tshz3
Bcor	Fbp2	Mc4r	Rcor2	Tsn
Bcr	Fbrsl1	Mcc	Rd3	Tspan11
Bdh1	Fbxl17	Mchr1	Reep1	Tspan12
Bdkrb2	Fbxl2	Mctp1	Reep2	Tspan14
Bend5	Fbxl4	Mctp2	Reep3	Tspan32
Bfsp2	Fbxl7	Mdfic	Refbp2	Tspan5

Gene name				
Bhlha9	Fbxo32	Mdga1	Reln	Tspan9
Bhlhe40	Fbxo47	Mdga2	Repin1	Tspyl1
Bicd2	Fbxo48	Mecr	Reps2	Tssc1
Bloc1s1	Fbxw11	Med10	Rere	Tstd2
Bmf	Fbxw4	Med13l	Rerg	Ttc1
Bmp6	Fchsd2	Med14	Ret	Ttc12
Bmp7	Fcnb	Med16	Rev1	Ttc17
Bmper	Fermt1	Med27	Rev3l	Ttc27
Bmpr1a	Fermt2	Med28	Rfc2	Ttc3
Bmpr1b	Fert2	Med30	Rffl	Ttc30a1
Bmpr2	Fez1	Med31	Rfk	Ttc33
Bnc2	Fezf2	Med4	Rftn2	Ttc37
Boc	Fgd3	Mef2a	Rfx2	Ttc39a
Bod1	Fgd4	Mef2c	Rfx3	Ttc39b
Bod1l	Fgf1	Mef2d	Rfx7	Ttc39c
Bpgm	Fgf11	Megf10	Rfx8	Ttc7
Bpi	Fgf12	Megf9	Rg9mtd3	Ttc9
Bptf	Fgf18	Meig1	Rgag4	Ttll11
Brd7	Fgf20	Meis1	Rgl1	Ttll3
Brsk2	Fgf21	Meis2	Rgma	Ttll5
Bst2	Fgfr1	Memo1	Rgmb	Ttll7
Btaf1	Fgfr4	Meox1	Rgnef	Ttpa
Btbd1	Fgfrl1	Meox2	Rgr	Tub
Btbd11	Fggy	Mertk	Rgs14	Tuba1a
Btbd16	Fgl1	Metap2	Rgs16	Tuba1b
Btbd17	Fgr	Mett5d1	Rgs17	Tubb2a
Btbd3	Fhdc1	Mettl11b	Rgs20	Tubb2b
Btbd9	Fhit	Mex3b	Rgs22	Tubg1
Btd	Fhl3	Mex3c	Rgs3	Tulp1
Btg1	Fhl4	Mfap3	Rgs7	Tulp3
Btg2	Fign	Mfge8	Rgs7bp	Tulp4
Btla	Fkbp11	Mfhas1	Rgs8	Twsg1
Bzw2	Fli1	Mfsd6	Rhbdd3	Txndc2
C030046E11Rik	Flnb	Mgat4a	Rhbdl3	Txndc3
C130022K22Rik	Flnc	Mgat5	Rhbg	Txndc8
C130039O16Rik	Flrt1	Mgat5b	Rhcg	Txnip
C1d	Flrt2	Mgst1	Rheb	Txnl1
C1ql1	Flrt3	Mgst2	Rhob	Txnrd2
C1qtnf1	Flywch2	Mib1	Rhobtb1	Tyr
C1qtnf5	Fmn1	Micalcl	Rhobtb3	Tyro3
C1qtnf7	Fmn2	Micall1	Rhof	Tyrp1
C1qtnf9	Fmnl2	Mid1ip1	Rhoh	Tyw3
C230052I12Rik	Fmnl3	Midn	Rhoq	Uaca
C330007P06Rik	Fnbp1	Mif4gd	Ric8b	Uap1
C530008M17Rik	Fndc1	Minpp1	Rimbp3	Uba5
C77080	Fndc3b	Mitf	Rimkla	Ubash3b
C78339	Fos	Mki67	Rims1	Ubc
C8b	Foxa2	Mkl2	Rin2	Ube2e1
C9	Foxc1	Mkln1	Rin3	Ube2e2
CK137956	Foxd3	Mkrn1	Ripk1	Ube2e3
Cabin1	Foxf1a	Mlf1ip	Ripk2	Ube2g2
Cables1	Foxg1	Mll1	Rit2	Ube2u
Cabp7	Foxi1	Mll5	Rlbp1	Ube2v1
Cabyr	Foxk1	Mllt3	Rmi1	Ube4b
CacnB2	Foxl1	Mllt4	Rnase1	Ubl3
Cacna1a	Foxn2	Mlxip	Rnase13	Ubl4b

Gene name				
Cacna1b	Foxo1	Mmaa	Rnaseh2b	Ubtf
Cacna1h	Foxo6	Mmab	Rnd3	Uchl4
Cacna2d1	Foxp1	Mmadhc	Rnf112	Uck2
Cacna2d4	Foxp2	Mmd2	Rnf114	Ugcg
Cacnb4	Foxp4	Mme	Rnf122	Ulk4
Cacng1	Frmd4a	Mmp15	Rnf144a	Umps
Cacng3	Frmd4b	Mmp16	Rnf144b	Unc13c
Cacng4	Frmd6	Mmp28	Rnf150	Unc5c
Cacng5	Frmd8	Mms19	Rnf152	Ung
Cadm1	Frmpd1	Mms22l	Rnf182	Upb1
Cadm2	Fry	Mn1	Rnf19b	Upp2
Calb2	Fryl	Mocs2	Rnf214	Uqcr10
Calcoco1	Fsbp	Mogat2	Rnf216	Urm1
Calcrl	Fscn1	Mon1b	Rnf4	Urod
Calm1	Fsd1l	Mon2	Rngtt	Ush2a
Calm2	Fsd2	Morc2a	Rnmtl1	Usp10
Calr4	Fshr	Morc3	Robo1	Usp12
Camk1d	Fsip1	Morn3	Robo2	Usp24
Camk1g	Fstl1	Moxd1	Rora	Usp25
Camk2a	Fstl4	Mpa2l	Rorb	Usp3
Camk2b	Fth1	Mpdz	Rpa1	Usp31
Camk2d	Ftl2	Mpg	Rpe	Usp38
Camk2n1	Fto	Mphosph9	Rph3a	Usp44
Camkk2	Fxn	Mpp3	Rpia	Usp46
Camsap1l1	Fyn	Mpp6	Rpl14	Usp6nl
Cand1	Fzd1	Mpped1	Rpl15	Ust
Cap2	Fzd4	Mpped2	Rpl27a	Utp18
Capg	Fzd6	Mprip	Rpl29	Utp6
Capn12	Fzd7	Mpzl2	Rpl37a	Vac14
Capn13	Fzd9	Mr1	Rpl38	Vamp3
Capn5	G0s2	Mras	Rplp1	Vangl1
Capns2	G2e3	Mrc2	Rprd1b	Vash1
Car12	G3bp2	Mreg	Rprm	Vash2
Car5a	G6pc3	Mro	Rps10	Vav2
Car6	Gab1	Mrpl33	Rps14	Vav3
Car8	Gab2	Mrpl39	Rps24	Vcl
Car9	Gabra2	Mrpl48	Rps29	Vdac1
Card11	Gabrg1	Mrps10	Rps6ka2	Vdac2
Card9	Gadd45a	Mrps18a	Rptor	Vegfa
Casc1	Gadd45b	Mrps23	Rpusd4	Veph1
Casp12	Gadd45g	Mrps28	Rragc	Vgll2
Casp14	Gadl1	Msh6	Rreb1	Vgll3
Casp3	Galc	Msi2	Rrm2	Vgll4
Casz1	Galm	Msl3l2	Rrp7a	Vldlr
Cat	Galnt10	Msrb3	Rsl24d1	Vmn1r3
Cav2	Galnt2	Msx1	Rtkn	Vmn1r4
Cbara1	Galnt4	Mtap1a	Rtkn2	Vmn1r64
Cbfa2t2	Galnt7	Mtap1b	Rtn3	Vmn2r18
Cbfa2t3	Galntl1	Mtap2	Rtn4	Vps13b
Cbln1	Galntl2	Mtap4	Rtn4rl1	Vps13c
Cbln4	Galntl4	Mtap6	Rufy3	Vps37b
Cbr4	Galntl5	Mtap7	Rundc1	Vps45
Cbwd1	Galr3	Mtbp	Runx1	Vps53
Cbx4	Gap43	Mtch1	Runx1t1	Vps54
Cbx5	Gapdh	Mthfd1l	Rusc2	Vps8
Cbx7	Gapvd1	Mtmr10	Rxfp2	Vrk1

Table A1 1

Gene name				
Ccbe1	Garnl3	Mtmr2	Rxra	Vstm2a
Ccdc11	Gas1	Mtmr6	Rxrg	Vstm2b
Ccdc121	Gas2l3	Mtr	Rybp	Vstm2l
Ccdc132	Gas6	Mtss1	Ryk	Vti1a
Ccdc134	Gas7	Mtss1l	Ryr3	Vwde
Ccdc136	Gatm	Muc20	S100a10	Vwf
Ccdc141	Gatsl2	Muc3	S100a16	Wac
Ccdc148	Gbe1	Mudeng	S100a4	Was
Ccdc30	Gbf1	Murc	S100a6	Wasf1
Ccdc41	Gbx2	Mus81	S1pr2	Wasf2
Ccdc42	Gca	Mxra7	Sae1	Wasf3
Ccdc50	Gch1	Myadm	Safb2	Wdfy2
Ccdc54	Gclc	Mybpc1	Sag	Wdr20b
Ccdc6	Gcnt1	Mybph	Sall1	Wdr26
Ccdc69	Gcnt2	Myc	Sall3	Wdr27
Ccdc70	Gcom1	Mycn	Samd5	Wdr37
Ccdc8	Gdf10	Myh14	Samd7	Wdr38
Ccdc81	Gdf6	Myh7b	Sap30l	Wdr4
Ccdc83	Gdi2	Myh9	Sardh	Wdr44
Ccdc85c	Gemin8	Myl1	Sars	Wdr66
Ccdc86	Gfod1	Mylk	Sash1	Wdr7
Ccdc88c	Gfpt2	Myo10	Satb1	Wdr72
Ccdc90a	Gfra1	Myo16	Scamp5	Wdr95
Ccdc90b	Gfra2	Myo18a	Scand1	Wdsub1
Ccdc91	Gga1	Myo18b	Scap	Whrn
Ccin	Ggh	Myo1e	Scarb1	Whsc1l1
Ccl22	Ggt5	Myo3a	Scarb2	Wif1
Ccl25	Ggta1	Myo3b	Sccpdh	Wipf3
Ccl9	Ghrh	Myo5a	Scel	Wisp1
Ccna1	Gig18	Myo7a	Scfd2	Wisp3
Ccnd1	Gin1	Myog	Scg2	Wls
Ccnd2	Gja8	Myoz3	Scgb1a1	Wnt10a
Ccnd3	Gjb6	Myrip	Scgb1c1	Wnt2b
Ccnjl	Gjc3	Myst4	Scgn	Wnt5a
Ccnl1	Gjd2	Myt1	Scml4	Wnt7a
Ccr3	Gkn1	Myt1l	Scn3b	Wnt7b
Ccr6	Glcci1	Mzt1	Scn5a	Wrn
Ccrn4l	Glce	N4bp1	Scn8a	Wsb1
Cct6b	Gle1	N6amt1	Scrg1	Wscd1
Cct8l1	Glg1	Naa20	Scrn1	Wt1
Cd109	Gli2	Nacc2	Scx	Wwc1
Cd164	Gli3	Naif1	Scyl1	Wwox
Cd180	Glis1	Nalcn	Sdc1	Wwp2
Cd2	Glis3	Nanogpd	Sdc2	Wwtr1
Cd247	Glp1r	Nap1l1	Sdc3	X99384
Cd276	Glrb	Nap1l5	Sdccag8	Xkr9
Cd28	Glrx	Napa	Sdhaf1	Xpnpep2
Cd47	Glt25d2	Napepld	Sdk1	Xpo4
Cd69	Glt28d2	Napg	Sdk2	Xpo6
Cd81	Glud1	Narg2	Sdpr	Xrcc2
Cd82	Glul	Nat3	Sec14l1	Xrcc4
Cd83	Gm10391	Nat8	Sec14l4	Xrcc6bp1
Cd93	Gm1060	Nav1	Sec14l5	Xrn2
Cd96	Gm11744	Nav2	Sec16b	Xylt1
Cd97	Gm11818	Nav3	Sec22b	Yes1
Cdc25b	Gm12695	Nbas	Sec24b	Ykt6

Gene name				
Cdc42ep1	Gm12824	Nbr1	Sec61b	Ythdc2
Cdc42se1	Gm1337	Ncald	Sec61g	Ywhaq
Cdc42se2	Gm13695	Ncam1	Sel1l	Ywhaz
Cdc5l	Gm14483	Ncam2	Sema3a	Zbed3
Cdc6	Gm15319	Ncf2	Sema3b	Zbtb10
Cdca7	Gm1568	Nck2	Sema3d	Zbtb16
Cdh10	Gm1587	Nckap5	Sema4a	Zbtb20
Cdh11	Gm1631	Nckap5l	Sema4b	Zbtb43
Cdh13	Gm16378	Ncoa1	Sema4c	Zbtb7c
Cdh2	Gm17019	Ncoa3	Sema4d	Zc3h12c
Cdh20	Gm1965	Ncor2	Sema5a	Zc3h3
Cdh26	Gm239	Ncs1	Sema5b	Zcchc13
Cdh4	Gm3417	Ncstn	Sema6a	Zcchc2
Cdh5	Gm347	Ndfip1	Sema6d	Zcchc24
Cdh6	Gm3646	Ndn	Sep15	Zcchc6
Cdh8	Gm4340	Ndnl2	Sepp1	Zdhhc14
Cdhr3	Gm4906	Ndrg1	Sepsecs	Zdhhc19
Cdk14	Gm4937	Ndrg3	Sept11	Zdhhc20
Cdk17	Gm4951	Ndst4	Sept4	Zdhhc21
Cdk19	Gm4980	Ndufa10	Sept7	Zdhhc25
Cdk2ap1	Gm5134	Ndufa11	Sept9	Zdhhc7
Cdk5r1	Gm5136	Ndufa4	Serac1	Zdhhc9
Cdk5rap2	Gm5148	Ndufb9	Sergef	Zeb1
Cdk6	Gm527	Ndufv3	Serhl	Zeb2
Cdk8	Gm5382	Necab1	Serinc1	Zfand5
Cdkal1	Gm5415	Nedd4	Serinc5	Zfand6
Cdkl1	Gm5506	Nedd4l	Serp2	Zfat
Cdkn1a	Gm5820	Nedd9	Serpina12	Zfc3h1
Cdkn1c	Gm597	Negr1	Serpina3n	Zfhx2
Cdkn2aipnl	Gm628	Neil1	Serpinb5	Zfhx3
Cdkn2d	Gm6588	Nek2	Serpine2	Zfp106
Cdrt4	Gm672	Nek7	Serpine3	Zfp217
Cdv3	Gm6724	Nek9	Serpinh1	Zfp238
Cdyl	Gm6772	Nell2	Serpini2	Zfp280b
Ceacam1	Gm6878	Nenf	Sertad2	Zfp281
Ceacam2	Gm6924	Neo1	Sertad3	Zfp330
Cebpd	Gm7073	Nes	Sesn3	Zfp362
Cebpg	Gm71	Neto1	Setd5	Zfp365
Cecr2	Gm7325	Neu4	Sez6	Zfp366
Cecr5	Gm766	Neurl1a	Sez6l	Zfp36l1
Celf2	Gm813	Neurl1b	Sf3b4	Zfp36l2
Cenpc1	Gm815	Neurod4	Sfi1	Zfp385b
Cenpo	Gm8882	Neurod6	Sfmbt2	Zfp41
Cep164	Gm9104	Nf2	Sfrp2	Zfp423
Cep170	Gm973	Nfam1	Sfswap	Zfp445
Cep68	Gm9733	Nfasc	Sfxn1	Zfp457
Cer1	Gm9758	Nfatc2	Sfxn5	Zfp462
Cerk	Gm9880	Nfatc3	Sgcz	Zfp474
Ces5a	Gm9920	Nfe2l2	Sgip1	Zfp488
Cetn3	Gm9934	Nfe2l3	Sgk1	Zfp516
Cfdp1	Gm9961	Nfia	Sgk2	Zfp521
Cfl2	Gm9992	Nfib	Sgms1	Zfp532
Cga	Gmds	Nfic	Sh2b3	Zfp536
Cgnl1	Gmpr	Nfil3	Sh2d1a	Zfp608
Chac1	Gna12	Nfix	Sh2d4a	Zfp609
Chchd3	Gna13	Nfkb2	Sh2d4b	Zfp664

Gene name				
Chchd8	Gnao1	Nfkbia	Sh2d5	Zfp69
Chd2	Gnaq	Nfkbiz	Sh2d6	Zfp703
Chd3	Gnat3	Nfyc	Sh2d7	Zfp704
Chd6	Gnaz	Ng23	Sh3bgrl	Zfp706
Chd7	Gnb2	Ngdn	Sh3bgrl2	Zfp740
Chd9	Gne	Ngly1	Sh3bp4	Zfp787
Chgb	Gng12	Nhlrc2	Sh3gl2	Zfp804a
Chic2	Gng13	Nhs	Sh3kbp1	Zfp827
Chit1	Gng2	Nhsl1	Sh3pxd2a	Zfp868
Chl1	Gng4	Nicn1	Sh3pxd2b	Zfp945
Chmp4c	Gnpda1	Nin	Sh3rf1	Zfp957
Chmp6	Gnpda2	Ninj1	Sh3rf3	Zfpm1
Chn2	Golga5	Ninj2	Sh3tc2	Zfpm2
Chrac1	Golga7b	Nipal2	Shb	Zfr
Chrd	Golim4	Nipsnap3a	Shc4	Zfyve21
Chrdl1	Golm1	Nkain2	She	Zfyve28
Chrm1	Golph3	Nkain3	Shf	Zhx2
Chrm2	Golph3l	Nkd1	Shh	Zic1
Chrm3	Golt1a	Nkiras2	Shisa2	Zic4
Chrm4	Gorasp2	Nkx6-3	Shisa4	Zmiz1
Chrna4	Gp9	Nlgn1	Shox2	Zmiz2
Chst1	Gpc1	Nln	Shq1	Zmym2
Chst11	Gpc6	Nmnat2	Shroom3	Zmynd11
Chst2	Gpcpd1	Nmu	Shroom4	Zmynd8
Chst3	Gpd1	Noc3l	Siae	Znrf1
Chst4	Gpd2	Noc4l	Siah1a	Znrf2
Chst5	Gphb5	Nol11	Siah3	Znrf3
Chst7	Gphn	Nol9	Siglech	Zpld1
Chst9	Gpm6a	Nos1ap	Sik1	Zscan2
Chsy1	Gpm6b	Nos2	Sik2	Zswim4
Chsy3	Gpnmb	Nostrin	Sik3	Zswim6
Cidea	Gpr101	Notch1	Sil1	Zxdc
Cirbp	Gpr110	Notch2	Sipa1l1	rp9
Cit	Gpr123	Nova1	Sipa1l2	

Table A1 2 Genes that are bound by Ascl1 and downregulated in quies NSCs (from Martynoga et al., 2013)

Gene name				
1700011E24Rik	Csrnp3	Igfbp4	Pdzrn4	Slco1c1
1700017B05Rik	D8Ertd82e	Igfbp5	Peg12	Slit1
1700025G04Rik	Dcc	Igsf21	Pemt	Smad5
2610002I17Rik	Dctd	Il17d	Pgap1	Smc2
2610021K21Rik	Ddc	Impdh1	Phc2	Smtn
2610039C10Rik	Ddr2	Inhbb	Pinx1	Smyd2
2610528E23Rik	Ddx11	Insm1	Pion	Sncaip
3110035E14Rik	Ddx18	Itgb1bp2	Pkig	Snrpe
3830431G21Rik	Dgkd	Itpr2	Pld1	Sox11
4930427A07Rik	Dgkg	Ivns1abp	Pnmal1	Sox3
5330437I02Rik	Dhrs2	Jag1	Poc1a	Sox6
5730559C18Rik	Disc1	Josd2	Poc1b	Sox8
6230409E13Rik	Dlgap3	Kank1	Pola2	Spata24
6530418L21Rik	Dll1	Kbtbd8	Polg	Specc1
A630055G03Rik	Dll3	Kcnab2	Polr1a	Spred1
A730011L01Rik	Dmp1	Kcnc1	Polr3k	Spry1
AW146020	Dmrtb1	Kcnip3	Pom121	Spry2
Abcc12	Dnmt1	Kcnj10	Pop1	Spry4
Acaca	Dnmt3b	Kcnj2	Pou3f1	Spsb4
Acap3	Dock10	Kcnk2	Ppargc1b	Srgap2
Acer3	Dpf3	Kcnma1	Ppp1r14b	Srl
Acly	Dpp6	Kdm2b	Ppp1r14c	Srsf10
Acss1	Dscam	Kif14	Ppp2r5c	Srsf3
Ada	Dtl	Kif20b	Ppp5c	Srsf7
Adam12	Dusp4	Kirrel3	Prdm11	Stard9
Adam19	Dusp6	Kitl	Prep	Stc1
Adamts12	Dusp7	Klf12	Prkcb	Stim2
Adamts17	E2f1	Klhl13	Prkg2	Stk33
Adamts6	E2f3	Klhl23	Prokr1	Sykb
Adamts7	E2f7	Klhl5	Prr18	Tacc3
Amz1	Ebna1bp2	Ky	Prr5	Tbc1d16
Apcdd1	Edil3	Lamb1	Psat1	Tbc1d30
Apln	Ednrb	Lbr	Pstpip2	Tcf12
Areg	Eefsec	Lfng	Ptch1	Tcf4
Arhgap18	Efcab5	Lgr5	Ptgds	Tecta
Arhgap24	Egfl7	Lhx2	Ptprb	Tex14
Arhgap26	Egfr	Limd1	Ptprj	Tgfbr2
Arl6ip6	Elac2	Lipg	Ptpro	Tgif2
Arpp21	Elavl2	Lmcd1	Ptprs	Thrsp
Ascl1	Elavl4	Lmnb1	Ptprz1	Tk1
Atp1a2	Elovl6	Lmo1	Rab17	Tle1
Atp6v1e2	Enpp1	Lmo3	Rab3c	Tle4
Atp9a	Epb4.1	Lmo7	Rab8b	Tmc6
Axin2	Eps8	Lnp	Racgap1	Tmem149
B3galt5	Erh	Lrrc40	Rad51l1	Tmem173
B3gat1	Etv1	Lrrc8b	Raet1d	Tmem182
BC027231	Etv4	Lrrc9	Raly	Tmem209
BC030867	Etv5	Lrrfip1	Rap1gap	Tmem39b
BC052040	Eya2	Lrrn1	Rapgef3	Tmem44
Bahcc1	Fabp7	Lsm6	Rasa3	Tmem51
Bai2	Fam105a	Ltbp4	Rassf2	Tmod2
Bcat1	Fam122b	Ly75	Rassf3	Tmpo
Bcor	Fam174b	Lyn	Rcor2	Tmtc2

Gene name				
Bend5	Fam49b	Lypd6	Repin1	Tnfsf18
Bmp7	Fam78a	Magi1	Rerg	Tox2
Brsk2	Fbl	Map2k6	Rfc2	Tox3
Btbd17	Fbln1	Map4k4	Rfx3	Tppp
Btla	Fbln2	Mchr1	Rfx8	Tra2b
Bzw2	Fbp1	Mgat5b	Rgs7bp	Traf3ip2
C530008M17Rik	Fbxo48	Micalcl	Rhoq	Traf4
Calm1	Fgr	Mlf1ip	Rimbp3	Trib2
Camk1g	Fign	Mmd2	Rimkla	Trim67
Camk2a	Flrt3	Mmp15	Rlbp1	Trit1
Camk2b	Foxf1a	Morc2a	Rnaseh2b	Trmt61a
Camkk2	Foxp4	Mphosph9	Rnf122	Trp53i11
Car8	Frmd4a	Mpp3	Rnf144a	Tsga14
Ccdc141	Frmd4b	Mpped2	Rpl14	Tspan11
Ccdc69	Fyn	Mtbp	Rpl15	Tspan5
Ccna1	Fzd9	Mthfd1l	Rps14	Ttc3
Ccnd1	G2e3	Myc	Rrm2	Ttc37
Ccnjl	Gabrg1	Myh14	Rtkn2	Ttc7
Cd276	Galnt7	Myo18b	Ryr3	Ttpa
Cd82	Galntl1	Nap1l1	Scarb1	Tyro3
Cdc25b	Gas2l3	Nav3	Scml4	Ubash3b
Cdc6	Gas7	Ncald	Sdpr	Umps
Cdca7	Gfpt2	Nckap5l	Sec16b	Ung
Cdh4	Glcci1	Nek2	Sema4b	Usp10
Cdk2ap1	Gm10391	Nes	Sema4c	Usp3
Cdk6	Golph3	Neurl1a	Sema5a	Usp44
Cdk8	Gpr30	Nfia	Sema5b	Usp6nl
Cdkn1c	Gpsm1	Nfib	Sez6l	Utp18
Cdkn2aipnl	Gpt2	Noc4l	Sfrp2	Vash1
Cecr2	Grb10	Nol11	Sfxn1	Vash2
Chd3	Grip1	Nol9	Sgms1	Vav3
Chd7	Grm5	Nova1	Sh3bgrl2	Vrk1
Chn2	H2afy2	Nptx1	Sh3gl2	Wasf1
Chst7	Hap1	Nrk	Shc4	Wdr4
Chsy1	Hectd2	Ntn1	Shq1	Wdr66
Cit	Hes6	Ntrk2	Skp2	Wipf3
Clic5	Hfm1	Nuf2	Slc12a2	Wisp3
Cmpk2	Hip1	Nusap1	Slc14a2	Wnt7a
Cmtm5	Hipk2	Nwd1	Slc1a1	Wnt7b
Cntn6	Hlx	Ogg1	Slc1a3	Wscd1
Cntrob	Hmga2	Ola1	Slc22a23	Xylt1
Col9a3	Hmgcr	Olig1	Slc25a18	Zbtb10
Coro1c	Hnrpdl	Olig2	Slc29a4	Zdhhc21
Crb1	Hrh1	Otoa	Slc38a3	Zeb1
Crispld2	Iffo1	Pcdh10	Slc4a4	Zfp41
Csda	Igf1r	Pcdh18	Slc6a1	Zfyve28
Cspg5	Igfbp3	Pde4b	Slc6a11	Zswim6

Table A1 3 Specification of GO terms of genes bound by Ascl1 and downregulated in quies NSCs (from Martynoga et al., 2013)

Term	Count	%	p-value	Genes
Regulation of phosphorylation	25	5.07	7.81E-07	HMGCR, TRIB2, SPRY4, SPRY2, SPRY1, DGKD, DGKG, RAPGEF3, SPRED1, PPP1R14C, PPP1R14B, MAP2K6, EGFR, VAV3, LYN, TGFBR2, PKIG, SYKB, KITL, CDC25B, CDKN1C, ZFYVE28, NRK, BMP7, IGFBP3
Phosphate metabolic process	44	8.92	4.71E-05	STK33, FGR, NEK2, BRSK2, PRKG2, DDR2, D8ERTD82E, TRIB2, CAMKK2, IGF1R, VRK1, CAMK2B, CAMK2A, MAP2K6, PTPRB, PTPRJ, EGFR, TYRO3, CAMK1G, LYN, PTPRZ1, TGFBR2, CDK8, PTPRS, SYKB, CDK6, PTPRO, CDC25B, PRKCB, MAP4K4, DUSP4, TEX14, CCND1, EYA2, FYN, ATP6V1E2, HIPK2, NTRK2, NRK, CIT, IGFBP3, DUSP7, DUSP6, PPP5C
Regulation of kinase activity	17	3.45	5.10E-05	VAV3, HMGCR, PKIG, TGFBR2, SYKB, KITL, SPRY4, TRIB2, CDC25B, SPRY2, SPRY1, DGKD, DGKG, ZFYVE28, NRK, SPRED1, MAP2K6
Protein amino acid autophosphorylation	10	2.03	5.56E-05	EGFR, IGF1R, VRK1, LYN, FYN, NTRK2, CAMK2B, SYKB, CAMK2A, CAMKK2
Neuron differentiation	26	5.27	5.97E-05	DCC, BRSK2, JAG1, CDH4, SEMA5A, SLC1A3, CRB1, LHX2, ETV1, OLIG1, OLIG2, ETV4, DSCAM, KCNMA1, PTPRZ1, DLL1, GAS7, NTN1, SLIT1, CDKN1C, ASCL1, CLIC5, NTRK2, CIT, BMP7, WNT7A
Cell cycle	34	6.90	7.87E-05	E2F1, GAS2L3, E2F3, NEK2, E2F7, PRR5, DDX11, MTBP, CAMK2B, CCNA1, CAMK2A, LFNG, CDC6, ERH, NUF2, SKP2, NUSAP1, CDK6, 1700017B05RIK, RACGAP1, TACC3, HMGA2, SMC2, VASH1, 2610039C10RIK, CDC25B, CDKN1C, CCND1, CNTROB, RASSF2, KIF20B, CDK2AP1, CIT, CALM1
Sensory perception of mechanical stimulus	11	2.23	1.54E-04	KCNMA1, TECTA, SPRY2, CHD7, SLC1A3, SLC12A2, FYN, CLIC5, PGAP1, MYC, OTOA

Positive regulation of developmental process	17	3.45	1.82E-04	TGFBR2, SMAD5, CD276, SYKB, JAG1, CDH4, NTN1, VASH2, KITL, ADA, ASCL1, WNT7B, HLX, TGIF2, BMP7, IGFBP3, WNT7A
Pattern specification process	19	3.85	5.61E-04	PGAP1, SMAD5, TGFBR2, DLL3, DLL1, ZEB1, SEMA5A, PEG12, ASCL1, FOXF1A, SFRP2, LHX2, HIPK2, PTCH1, RFX3, BMP7, AXIN2, WNT7A, LFNG

Table A1 4 Genes that are bound by Ascl1 and enriched in aNSCs (from Codega et al., 2014)

Gene name				
2610039C10Rik	Cycs	Hap1	Nusap1	Slc38a1
2610109H07Rik	D19Bwg1357e	Hddc2	Odc1	Slc44a1
2610528E23Rik	D430041D05Rik	Hes6	Ola1	Slc4a7
2700060E02Rik	D8Ertd82e	Hmgb1	Papd7	Smarca5
4930427A07Rik	Dapk1	Hnrpll	Pbx3	Smc2
4930503L19Rik	Dclk2	Ift27	Pcdh18	Smyd2
A630055G03Rik	Dcx	Igfbpl1	Pdcd1	Sncaip
Adam12	Ddx20	Insm1	Peg12	Snd1
Ahcy	Dhx15	Ipo5	Pknox1	Snrnp40
Akt3	Dhx30	Iqgap1	Plcl2	Snrpe
Ankrd6	Dleu7	Kcnip3	Plrg1	Snx5
Apaf1	Dlgap3	Kdm2b	Plxna2	Socs3
Arl4c	Dll1	Kitl	Pola2	Sox11
Ascl1	Dll3	Lbr	Pold3	Sox4
Asnsd1	Dlx2	Lima1	Polr3k	Spata13
Asxl1	Dnmt1	Lmnb1	Pou3f4	Srsf3
Aven	Dpysl3	Lmo1	Ppil1	St3gal3
Bag1	Dscaml1	Lrfn4	Ppp1r14b	Stag1
Btg2	Dtl	Lrrfip1	Psmb1	Tacc3
Bzw2	E2f1	Lsm5	Ptprs	Tcf12
C230052I12Rik	E2f3	Lsm6	Rab8b	Tcf20
C530008M17Rik	E2f7	Maged1	Racgap1	Tead1
Casp3	E2f8	Magoh	Raly	Tfdp2
Cbx5	Edar	Map3k1	Rbm15	Tgif2
Ccdc86	Edc3	Marcks	Rbm24	Thoc7
Ccnd1	Efhd2	Med14	Rcbtb2	Tk1
Ccnd2	Egfr	Mex3b	Rfc2	Tmem2
Cd276	Eif3f	Mn1	Rgs16	Tmpo
Cdc6	Eif3h	Mphosph9	Rhbdl3	Tmsb10
Cdca7	Elavl2	Mrps10	Rnaseh2b	Tmsb4x
Cdh4	Elavl4	Mrps23	Rnf4	Tnfrsf21
Cdk2ap1	Enc1	Msh6	Rngtt	Tra2b
Cdk5r1	Epb4.1	Mtap1b	Rpa1	Trim33
Cdk6	Epha4	Mtap2	Rpia	Trim67
Cebpg	Ephb2	Mycn	Rpl14	Tsn
Celf2	Erh	Myt1	Rpl15	Ttc3
Cenpc1	Etaa1	Nap1l1	Rpl37a	Txnip
Cenpo	Fam108c	Ncald	Rps10	Ubtf
Chd3	Fam110a	Ncor2	Rps29	Uck2
Chd7	Fam64a	Nedd4l	Rrm2	Umps
Cit	Fbl	Nek2	Rtkn2	Usp10
Clic4	Flnb	Nell2	Sae1	Vamp3
Cnih4	Fos	Nes	Sec61g	Vash1
Cntrob	Fscn1	Nfia	Sez6	Vps37b
Coro1c	G2e3	Nfib	Sh3bgrl	Vrk1
Crmp1	Gab2	Nfix	Shf	Zc3h12c
Csda	Gng2	Nin	Sipa1l1	Zfp238
Csk	Golm1	Noc4l	Skp2	Zfp41
Csnk1e	Gse1	Nsg2	Slc25a5	Zfp462
Ctnnb1	Gsx1	Nuf2	Slc29a4	Zmiz1

Table A1 5 Specification of GO terms of genes bound by Ascl1 and enriched in aNSCs (from Codega et al., 2014)

Term	Count	%	p-value	Genes
DNA replication	13	5.24	1.72E-06	CDC6, DTL, PAPD7, NFIX, POLA2, TK1, RPA1, POLD3, RFC2, RRM2, A630055G03RIK, NFIA, NFIB
Cell cycle	26	10.48	1.84E-06	E2F1, E2F3, NEK2, E2F7, E2F8, RPA1, TFDP2, STAG1, TXNIP, CDC6, ERH, SKP2, PAPD7, NUF2, NUSAP1, CDK6, TACC3, RACGAP1, SMC2, VASH1, 2610039C10RIK, CCND1, CCND2, CNTROB, CDK2AP1, CIT
mRNA processing	15	6.05	2.26E-05	RALY, TRA2B, PPIL1, LSM6, MAGOH, HNRPLL, RNGTT, PLRG1, DHX15, LSM5, CELF2, THOC7, SNRNP40, DDX20, SNRPE
Forebrain development	12	4.84	2.62E-05	E2F1, EGFR, ASCL1, DLX2, GSX1, POU3F4, 2610109H07RIK, APAF1, TACC3, NCOR2, CTNNB1, NFIB
Regulation of transcription	55	22.18	3.15E-05	E2F1, E2F3, ZFP41, E2F7, E2F8, CTNNB1, KCNIP3, CBX5, MAGED1, FOS, CDCA7, SND1, ZFP238, LRRFIP1, DDX20, SOX11, MED14, HES6, CSDA, MYCN, ASCL1, KDM2B, ZFP462, BTG2, TRIM33, ZMIZ1, SMARCA5, TGIF2, HMGB1, SOX4, NFIX, MYT1, TCF20, CHD7, POU3F4, TFDP2, CHD3, TXNIP, POLR3K, CEBPG, ASXL1, GSX1, TEAD1, DLX2, PKNOX1, RNF4, UBTF, DNMT1, TMPO, PBX3, TCF12, RBM15, NFIA, NCOR2, NFIB
Regulation of RNA metabolic process	41	16.53	4.93E-05	E2F1, HMGB1, E2F3, E2F7, E2F8, SOX4, NFIX, MYT1, HNRPLL, CTNNB1, CBX5, KCNIP3, MAGED1, FOS, TFDP2, POU3F4, ZFP238, DDX20, TXNIP, SOX11, CEBPG, GSX1, TEAD1, MED14, HES6, CSDA, MYCN, ASCL1, DLX2, PKNOX1, ZFP462, ZMIZ1, SMARCA5, DNMT1, TGIF2, PBX3, RBM15, TCF12, NFIA, NCOR2, NFIB

Positive regulation of nitrogen compound metabolic process	21	8.467	5.98E-05	E2F1, HMGB1, E2F3, CEBPG, SOX11, GSX1, TEAD1, SOX4, MED14, NFIX, HNRPLL, CTNNB1, FOS, ASCL1, DLX2, PKNOX1, ZFP462, ZMIZ1, RBM15, NFIA, NFIB
DNA metabolic process	18	7.26	1.06E-04	CDC6, MSH6, DTL, C230052I12RIK, PAPD7, NFIX, POLA2, TK1, POLD3, RPA1, CASP3, RFC2, RRM2, DNMT1, A630055G03RIK, APAF1, NFIA, NFIB
Neuron differentiation	17	6.85	1.85E-04	CDK5R1, MTAP2, GSX1, DLL1, CDH4, EPHB2, EPHA4, ASCL1, DLX2, BTG2, BAG1, MTAP1B, POU3F4, 2610109H07RIK, PBX3, CIT, DCX

Table A1 6 Genes that are bound by Ascl1, downregulated in quies NSCs (Martynoga et al., 2013) and enriched in aNSCs (Codega et al., 2014)

Gene name				
2610039C10Rik	Coro1c	Hes6	Nusap1	Smc2
2610528E23Rik	Csda	Insm1	Ola1	Smyd2
4930427A07Rik	D8Ertd82e	Kcnip3	Pcdh18	Sncaip
A630055G03Rik	Dlgap3	Kdm2b	Peg12	Snrpe
Adam12	Dll1	Kitl	Pola2	Sox11
Ascl1	Dll3	Lbr	Polr3k	Srsf3
Bzw2	Dnmt1	Lmnb1	Ppp1r14b	Tacc3
C530008M17Rik	Dtl	Lmo1	Ptprs	Tcf12
Ccnd1	E2f1	Lrrfip1	Rab8b	Tgif2
Cd276	E2f3	Lsm6	Racgap1	Tk1
Cdc6	E2f7	Mphosph9	Raly	Tmpo
Cdca7	Egfr	Nap1l1	Rfc2	Tra2b
Cdh4	Elavl2	Ncald	Rnaseh2b	Trim67
Cdk2ap1	Elavl4	Nek2	Rpl14	Ttc3
Cdk6	Epb4.1	Nes	Rpl15	Umps
Chd3	Erh	Nfia	Rrm2	Usp10
Chd7	Fbl	Nfib	Rtkn2	Vash1
Cit	G2e3	Noc4l	Skp2	Vrk1
Cntrob	Hap1	Nuf2	Slc29a4	Zfp41

Table A1 7 Specification of GO terms of genes bound by Ascl1, downregulated in quies NSCs (Martynoga et al., 2013) and enriched in aNSCs (Codega et al., 2014)

Term	Count	%	p-value	Genes
Cell cycle	19	20.21276596	3.83E-09	E2F1, CDC6, E2F3, ERH, NEK2, E2F7, NUF2, SKP2, NUSAP1, CDK6, RACGAP1, TACC3, SMC2, VASH1, 2610039C10RIK, CCND1, CNTROB, CDK2AP1, CIT
Mitosis	7	7.446808511	6.25E-04	CDC6, NEK2, NUF2, NUSAP1, CIT, SMC2, 2610039C10RIK
Regulation of neurogenesis	6	6.382978723	8.03E-04	ASCL1, DLL1, TGIF2, CIT, CDH4, TTC3
Regulation of cell proliferation	10	10.63829787	0.002743904	EGFR, E2F3, CDCA7, E2F7, CD276, RTKN2, DNMT1, KITL, VASH1, NFIB
Cytoskeleton organization	7	7.446808511	0.0091606	CORO1C, EPB4.1, CNTROB, NUF2, NUSAP1, RACGAP1, TACC3

Appendix 2

Interactions between precursors cells in the DG and components of the neurogenic niche have been shown to be of vital importance for the regulation of stem cell self-renewal and differentiation (Doetsch, 2003). In the SVZ, functional interactions between blood vessels and stem cells have been uncovered (Ottone et al., 2014). Less is known about the nature of these interactions in the DG. Blood-borne factors are known to act on and regulate neuronal precursors (Goldberg and Hirschi, 2009). However, whether neuronal proliferation and neurogenesis acts on endothelial cells to regulate angiogenesis or blood vessel density is not known. A study by Palmer and colleagues (2000) reported precursors in the SGZ of rats to be closely associated with blood vessels. Moreover, they showed that 37% of DG BrdU immunoreactive cells after a 2-hour pulse were of the endothelial lineage. Together, these results suggested the possibility of a functional interaction between neurogenesis and angiogenesis in the rodent hippocampus. To test this hypothesis we first performed BrdU administration experiments to characterise the angiogenic population in the DG (Figure A2 1). Next, we hypothesised that in the case of interactions between neural and endothelial precursors taking place in the DG, a disruption in the neural precursor pool, would also result in a disruption of angiogenesis, and we tested this by examining endothelial cell division in *WT* and *Ascl1*^{neo}*cKO* mice (Figure A2 2).

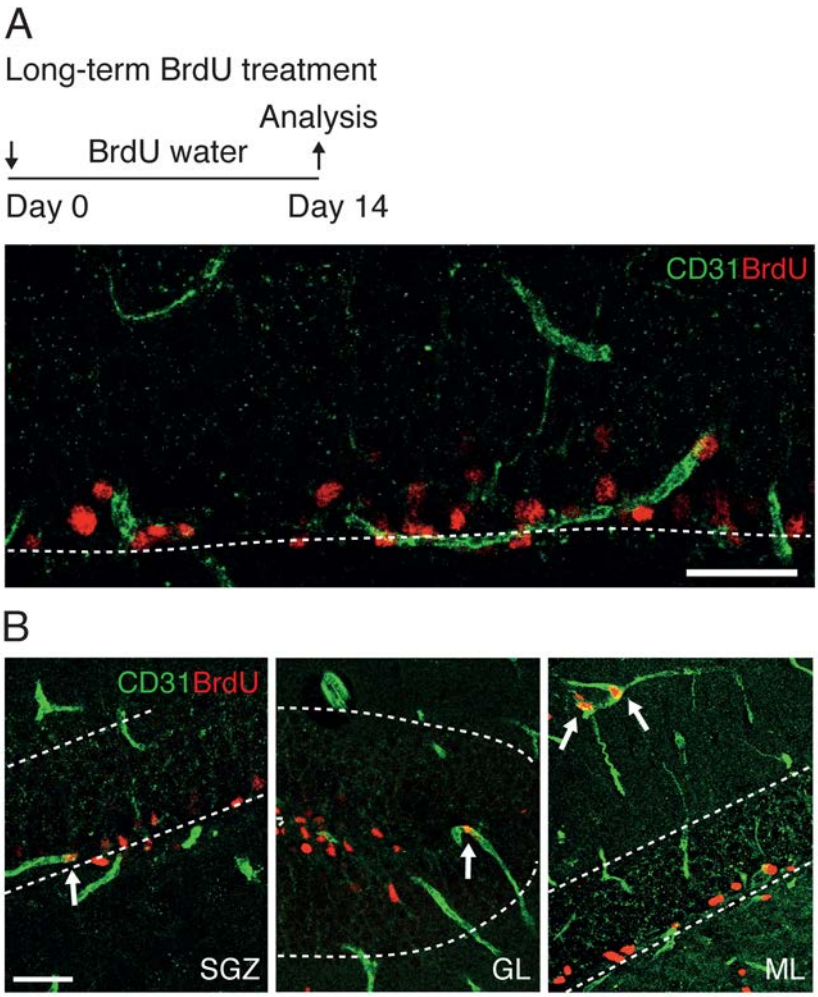

Figure A2 1 Only a small proportion of endothelial cells divides in the adult mouse hippocampus

(A) Administration of BrdU in the drinking water (0.2 mg/ml) for two weeks to 8 week-old *WT* mice to characterise the population of dividing endothelial cells in the hippocampus. Note that after a 2-hour BrdU pulse virtually no dividing endothelial cells were found in the hippocampus (not shown). Immunolabeling for BrdU and the endothelial marker CD31 shows that $BrdU^+$ cells are clustered in the vicinity of blood vessels, as expected from Palmer et al., 2000. **(B)** Colocalisation of BrdU and CD31 in different regions of the DG. A few $CD31^+$ $BrdU^+$ cells were found after two weeks of BrdU administration in the hippocampus, and these were either on the subgranular zone (SGZ), the granule layer (GL) or the molecular layer (ML). However, the number of these cells found did not correspond with the numbers observed in Palmer et al., 2000. In total, only approximately <1% of $BrdU^+$ cells also colocalised with CD31. This is unexpected, and would suggest that angiogenesis is not such a widespread phenomenon in the DG as reported before.

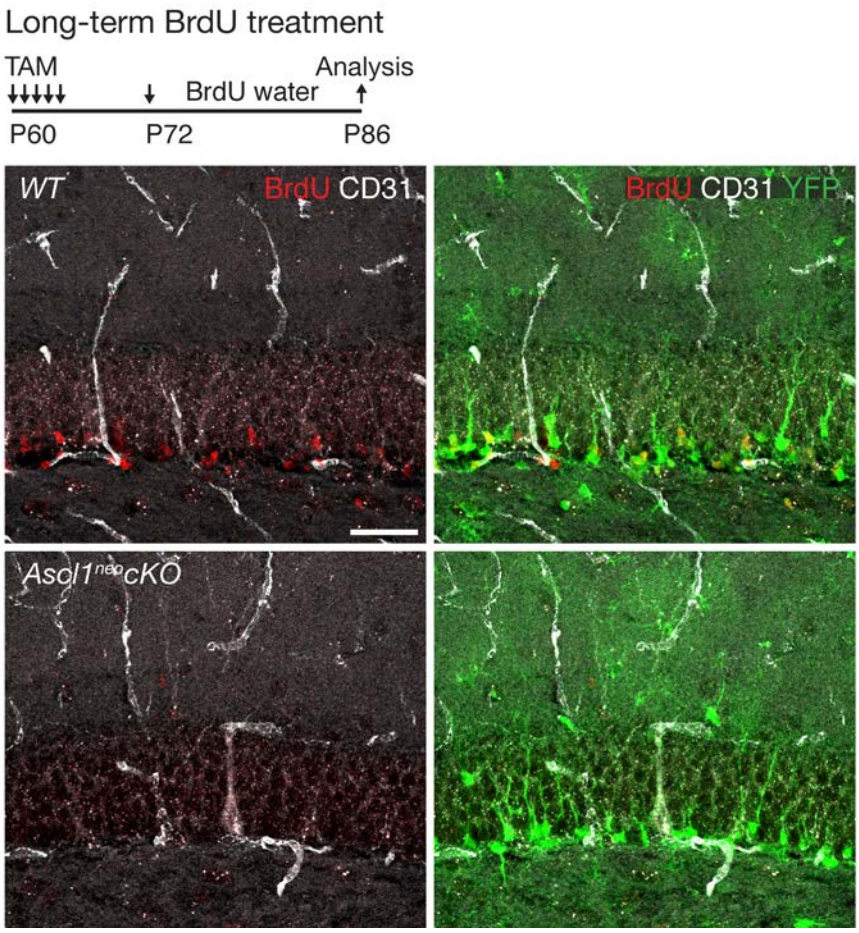

Figure A2 2 Investigation of functional interactions between neurogenesis and blood vessels

To study a possible functional cross-talk between neuronal precursors and blood vessels we administered BrdU in the drinking water (0.2 mg/ml) for two weeks to *WT* and *Ascl1^{neo}cKO* mice. *Ascl1^{neo}cKO* mice inactivate *Ascl1* in stem cells after TAM administration at P60. *Ascl1* deletion results in a block of stem cell proliferation and, consequently, neurogenesis (see Chapters 3 and 4). We hypothesised that neuronal precursors might regulate blood vessel density or angiogenesis in the DG. Labelling for BrdU, the endothelial marker CD31 and YFP to report recombination does not appear to show differences in blood vessel morphology or density between *WT* animals and animals that have lost neurogenesis. Moreover, no difference in angiogenesis was observed. These results, however, are not enough to discard functional interactions taking place between the neural and vascular systems in the hippocampus. Further characterisation and quantification of blood vessel quantity, density and branching will shed more light on this possibility. Scale bar = 40μm.